EFFECT OF PROCESSING ON QUALITY AND SAFETY OF CHERRY JUICE AND ITS BY-PRODUCTS

A Thesis

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Master of Science

by

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ABSTRACT

Cyanogenic Glycosides (CG) are compounds capable of producing hydrogen cyanide. Processed pits are potential saleable by-products if CG are removed. We evaluated the effect of processing on cherry juice and its by-products by measuring CG and quality attributes. After analysis by HPLC and ^{1}H-NMR, amygdalin was detected in kernels (13.6-23.0 mg/g), in flesh (0.07-0.09 mg/g) and in processed juice (0.06 mg/g). A significant reduction was observed in kernels after juicing (3.3 mg/g) and after 90 minutes of additional heat/acid treatment. Cherry pit oils were also extracted by expeller pressing yielding 2.9-4.4% and presenting comparable quality attributes but different aroma perception. No amygdalin was detected. After 12 weeks, sensory and quality parameters remained relatively stable. Melatonin was also determined by ^{1}H-NMR in different matrices. Quantification was effective between 1-100 ppm for aqueous solutions and supplements. No melatonin was detected in cherry products which may be associated with the detection limit.

BIOGRAPHICAL SKETCH

Belen was raised in Buenos Aires, Argentina. She was born celiac and since very young she has observed her mother trying to develop tasty and safe gluten free products for her. She grew up understanding the limitations of this disease and she naturally developed passion for food science. She got her five and a half year degree in Food Science and Technology at Universidad de Buenos Aires (UBA), being the valedictorian of her class. While studying she worked as a teaching assistant in the Analytical Chemistry Department of the School of Pharmacy and Biochemistry of Universidad de Buenos Aires. After graduation she worked as a laboratory analyst in a prestigious multinational company, SGS. Later on her career she took a position as Senior Corporate Quality and Food Safety Auditor and Consultant for Molinos Rio de la Plata, the Argentina's largest branded food products company. In 2015, Belen won the Fulbright Scholarship and the International Peace Scholarship and she joined the Padilla-Zakour research group as a Master Student. During her time at Cornell Belen participated in two product development competitions and she had an active participation in the Food Science Department. She served as the Secretary of the Food Science Graduate Student Organization, she was Founder and Vice-President of the Product Development Club and the M.S. Representative in the Curriculum Committee. Belen also received the KOSI Award in Food Science and she placed first in the Toxicology and Safety Evaluation Poster Competition at the 2017 Institute of Food Technologists Annual Meeting. After finishing her M.S. degree Belen will start working as a Management Trainee in J.R. Simplot. After one year of working in the U.S. she will be promoted to Junior Quality Manager in a new processing facility that the company will open in Argentina.

To Marcela Amadeo de Vila for always

being my inspiration and motivation.

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CHAPTER 1:

EFFECT OF JUICING AND POST PROCESS TREATMENT ON CYANOGENIC GLYCOSIDES CONTENT IN CHERRY PITS AND JUICE

1. Abstract

Pits of stone fruits such as cherries contain cyanogenic glycosides (CG). Processed pits are a potential saleable by-product of pitting and juicing operations if these compounds are removed. This study evaluated the effect of juice processing and additional heat/ acid conditions on the amount of CG on kernels, shells and flesh from two tart cherry cultivars and their corresponding pits and juice. A High Performance Liquid Chromatography (HPLC) method was developed to analyze these compounds and results were compared with Proton Nuclear Magnetic Resonance (^{1}H-NMR). Amygdalin was the only cyano-compound detected in kernels (Balaton 23.0, Montmorency 13.6 mg g^{-1}), in the flesh (Balaton 0.09, Montmorency 0.07 mg g^{-1}) and in the processed juice (0.06 mg g^{-1}). A significant reduction of this compound was observed in kernels after the juicing process (3.3 mg g^{-1}). A further amygdalin reduction was achieved after 90 minutes of exposure to the additional heat/acid treatment.

2. Introduction

The cherry market in the United States is led mainly by the production of tart cherry (*Prunus cerasus*) and sweet cherry (*Prunus avium*). While 76% of the sweet cherry production is allocated to the fresh market, 99% of the tart cherry harvest is processed (AgMRC, 2015). As a result of tart cherry processing operations, a large amount of pits are separated in the United States annually. In 2015, 222.6 million pounds of tart cherries were produced and for 2016, 309.1 million pounds are expected (USDA, 2016). However, finding a food-grade use for the tart cherry pits, resulting from the industrial operations, is a challenge due to the presence of cyanogenic glycosides.

Cyanogenic glycosides (CG) are plant secondary metabolites capable of liberating hydrogen cyanide (HCN) when hydrolyzed. These cyano-compounds are present in around 2500 species of the plant kingdom and may pose a health risk if ingested (Vetter, 2000). The toxicity of CG in plants is related to the potential concentration of HCN that can be produced if consumed (FAO/WHO, 2009). In 1940, a fatal case and other non-fatal intoxications were reported due to ingestion of chokecherry (*Prunus virginiana*) seeds. Patients experienced convulsion, dizziness, difficulty speaking and vomiting (Pijoan, 1942).

Amygdalin (D-mandelonitrile-β-D-gentebioside) and prunasin (D-Mandelonitrile-β-D-glucoside) are two CG present in the *Prunus* genus, which includes plums, cherries, peaches, apricots, nectarines and almonds (Janssen, Put, & Nout, 1997). These compounds can be hydrolyzed by the action of enzymes, β-glucosidases and α-hydroxynitrile lyases (Figure 1), present in the same plant and even in the human

microflora, posing a toxicity risk for humans (Barceloux, 2009; Bolarinwa, Orfila, & Morgan, 2014).

In 2011, the Joint FAO/WHO Expert Committee in Food Additives (JECFA) reported an acute reference dose value (ARfD) of 90 μg/kg bw/d cyanide equivalents and a provisional maximum tolerable daily intake (PMTDI) of 20 μg/kg bw/d (FAO/WHO, 2011). Although no specific limits are defined for pits of stone fruits, the Codex Alimentarius Commission defined limits for cassava products which are mostly consumed in Africa, representing a source of cyanogens (Cardoso et al., 2005). Limits below 50 mg/kg of hydrogen cyanide, 10 mg/kg of total hydrocyanic acid and 2 mg/kg as free hydrocyanic acid were set for sweet cassava, edible cassava flour and for Gari respectively (FAO/WHO, 2012). The Council of Europe Committee of Experts on Flavouring Substances (CEFS) also proposed maximum levels of CN^- in certain products. For instance, 0.5 mg kg^{-1} for stone fruits beverages and 2 mg kg^{-1} for canned stone fruits, stone fruit preserves and purees were recommended (EFSA, 2004). Bolarinwa, et al. (2014) reported concentrations of amygdalin of 2.68 and 3.89 mg g^{-1} in Black and Red cherry seeds respectively. If transformed to HCN or CN^- equivalents these products contain amounts above the previously defined limits.

Different strategies have been proposed over the years to remove or reduce the amount of CG in food products either by their solubility or by the production of hydrogen cyanide that can then be released. Soaking, fermenting, cooking, boiling and drying are some of the strategies that have been studied (Bradbury, 2006; Cardoso et al., 2005; Cardoso, Ernesto, Cliff, Egan, & Bradbury, 1998; Montagnac, Davis, & Tanumihardjo, 2009). However, most of this research has been conducted in cassava

products and there is little information about the processing impact within the *Prunus* genus. Voldrich and Kyzlink (1992) analyzed the effect of canning on the HCN content in different stone fruits. In addition, Eid and Schmidt (1978) studied the influence of the percentage of broken pits and other variables in the HCN content in cherry juice but there is no specific research found on the effect of juicing and post-processing treatments in tart cherry pits. Identifying procedures to reduce the amount of CG in fruit by- products is crucial to make food grade products from supplies with high concentrations of these compounds.

Several methods have been developed to identify and quantify CG in plants and food products. Picrate and acid hydrolysis methods have been used to determine the cyanide content in different food products (Bradbury, Egan, & Bradbury, 1999; Egan, Yeoh, & Bradbury, 1998; Haque & Bradbury, 2002). In addition, techniques involving the direct determination of the CG by HPLC or ^{1}H-NMR have been developed for different matrices but there are no studies that correlate these two methods (Berenguer-Navarro, Giner-Galván, Grané-Teruel, & Arrázola-Paternina, 2002; Gómez, Burgos, Soriano, & Marín, 1998; Santos Pimenta, Schilthuizen, Verpoorte, & Choi, 2014; Savic, Nikolic, Savic, Nikolic, & Stankovic, 2012; Swain, Li, & Poulton, 1992).

The objective of this study was to identify and quantify amygdalin and prunasin in kernels (seeds), shells (endocarps) and flesh of two tart cherry cultivars and in their corresponding processed pits and juices. The effect of the juicing process and a post-heat/acid treatment on these compounds was also evaluated. Samples were analyzed by HPLC and ^{1}H-NMR.

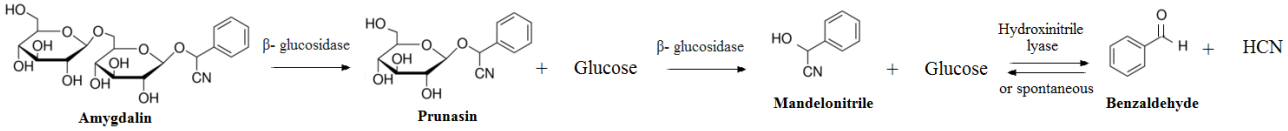

Figure 1: Amygdalin and prunasin enzymatic hydrolysis reaction.

3. Materials and methods

3.1. Reagents and Standards

Amygdalin (>97%) and Prunasin (>90%) standards were obtained from Sigma Aldrich (St. Louis, MO, USA). Methanol and Acetonitrile HPLC grade were from Fisher Scientific (Waltham, MA, USA). Deuterium Oxide (D_2O, D 99.9%) and 0.02% (W/V) Trimethylsilylpropanoic acid (TMSP, 2,2,3,3-D_4 98%) in D_2O (99.9%), TMSP, were obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Citric acid was from BDH®, VWR Analytical (Radnor, PA, USA).

3.2. Samples

All the cherries, pits and juices were obtained from a cherry juice manufacturing facility located in Northeastern New York. Two different tart cherry (*Prunus cerasus*) cultivars, Montmorency and Balaton, were picked approximately at 70 days from flowering and were kept frozen at -20^{o}C. Fresh (unprocessed) pits were obtained by pitting the thawed cherries in a Leifheit cherry stoner (Naussau, Germany). The flesh was separated and used as described in 3.12. Processed pits were a blend of these two cherry cultivars obtained from the juicing process described in 3.4. Pits were rinsed with abundant water and cracked to obtain kernels and shells. Immediately after, samples were freeze-dried (9 hour freezing-36 hours sublimation) in a Harvest Right Freeze Dryer (North Salt

Lake, UT, USA). Samples were stored in hermetically sealed glass jars at room temperature. Juice samples were obtained from the juicing process described in 3.4. All the experiments were conducted in triplicate.

3.3. Kernel and shell weights

Kernel and shells weights were recorded for each cultivar and for the processed pits blend. Thirty random samples of kernels and shells were weighed in each experimental replicate.

3.4. Juicing process

Montmorency and Balaton cherries were commercially processed by heating a blend of them up to 79ºC in a cooker. After reaching that temperature the juice was extracted and the cherry pits separated. The juice was packed in PET bottles and stored at 5ºC until used.

3.5. Heat and acid treatment in processed pits

One lot of the processed pits obtained from 3.4 was rinsed with water until all the pulp was removed. Ten grams of pits were weighed into centrifuge tubes and 40 g of a 2M citric acid solution were added. The centrifuge tubes were placed in a water bath at 85ºC with the lid loosely in place. Centrifuge tubes were removed from the bath in 30 min intervals for a total of 210 min (three tubes per time point). The tubes were allowed to cool down for 1 h and were then rinsed with water. Once rinsed, pits were cracked to obtain the kernels. Kernels were freeze-dried (9 h freezing – 36 h sublimation) and

stored in glass jars at room temperature. One sample was kept as control with no treatment.

3.6. Extraction of cyanogenic glycosides

The freeze-dried kernels and shells (3.2 & 3.5) were crushed with a mortar and pestle and a lab scale mill at 1725 rpm (General Electric, A-C Motor) respectively. Samples of 0.7 g were placed into a cellulose extraction thimble (Whatman^{TM}, 25 mm x 80 mm; Maidstone, UK) and were extracted in a Soxhlet apparatus at 65ºC with 100 ml of methanol for 6 h (Gómez et al., 1998). Solvent was evaporated and the extracts were kept in desiccators overnight. The kernel and shell extracts were diluted to 10 ml with a solvent mixture of acetonitrile and water - 75:25 v/v for kernels and 85:15 v/v for shells. Samples were sonicated in a Branson 2200 sonicator for 5 min. Three extractions were conducted in each component of each experimental replicate.

3.7. HPLC sample preparation

Only kernels and shells were analyzed by HPLC. For amygdalin determination in kernels, the diluted extracts obtained in 3.6 were further diluted with a solvent mixture (75% Acetonitrile: 25% Water) and mixed in a Vortex mixer for 10 s. Dilutions (1/20) were made from the fresh kernel extracts of each cultivar and from the processed kernel extract (1/10). For prunasin determination in kernels and for amygdalin determination in shells, the diluted extracts obtained in 3.6. were directly analyzed with no further dilution. All samples were passed through a 0.45 µm Nylon Syringe filter (VWR International, Radnor, PA, USA) before injection in the HPLC.

3.8. Calibration curves for HPLC

For the amygdalin calibration curve, a 1000 ppm (µg/ml) stock solution of amygdalin in mobile phase was prepared and further dilutions of 10, 20, 40, 60, 80 and 100 µg/ml were made. For the prunasin curve, a 40 ppm stock solution was prepared with mobile phase and the curve was constructed using 5 points containing 5, 10, 20, 30 and 40 µg/ml of prunasin. Conditions described in 3.9 and 3.10 were used to obtain these curves.

3.9. Chromatographic conditions for kernels

The method developed was based on the procedure described by Savic et al. (2012). An Agilent 1100 HPLC equipment with auto sampler, degasser, quaternary pump and Diode Array Detector set at 210 nm was used. The column was a Phenomenex Spherex NH_2 (250 x 4.60 mm, 5 µm) operating at 20^{o}C. Twenty µl of sample were injected each time and eluted under isocratic conditions with 1 ml/min flow rate. The mobile phase consisted of a mixture of acetonitrile and water (75:25, v/v). Chromatograms were analyzed with Agilent ChemStation Software (Rev. B. 04.03 (16)) and peaks were manually integrated.

3.10. Chromatographic conditions for shells

The same equipment and column described in 3.9. were used. Twenty µl of sample were injected each time and eluted under isocratic conditions with 1.2 ml/min flow rate. The mobile phase was a combination of acetonitrile and water (85:15, v/v) and the column

temperature was set at 30ºC. Chromatograms were analyzed with Agilent ChemStation Software (Rev. B. 04.03 (16)) and peaks were manually integrated.

3.11. HPLC recovery

Approximately 10 mg of amygdalin standard were placed into an extraction thimble and were extracted and analyzed by HPLC as described in 3.9. For prunasin the same procedure was followed but starting with 1 or 0.5 ml of a 35 ppm solution.

3.12. Flesh sample preparation for ^{1}H-NMR

The fresh flesh of the two different cherry cultivars, obtained after the pitting procedure in 3.1, were individually pressed in a Braun multipress automatic kitchen juicer (Aschaffenburg, Germany) to obtain a liquid product. The juice obtained was filtered through 0.215 mm coarse paper filters and analyzed as described in 3.13.

3.13. Quantification of amygdalin and prunasin by 1H-NMR

The method applied was based on the procedure described by Santos Pimenta et al. (2014). Between 20 and 40 µl of sample were placed in a capped vial with 300 µl of internal standard (TMSP) and 310 µl of deuterium oxide. Samples were kernel or shell extracts obtained in 3.6. or flesh or processed juice (3.4 and 3.12). The vials were agitated on a vortex mixer and the liquid was transferred to 5 mm high precision NMR tubes. Samples were analyzed on a Bruker AVANCE III HD 500 NMR instrument with an autosampler operating at a frequency of 500 MHz. The one-dimensional ^{1}H-NMR spectra were recorded at 25ºC with 3.2768 s acquisition time, a pulse angle of 90º and

a relaxation delay of 30 seconds. Sixty four scans were acquired for kernel and shell extracts. Five hundred and twelve and 1024 scans were acquired for the flesh and processed juice respectively. Results were analyzed with MestReNova (Version 11.0) by manually phasing, baseline correcting and integrating the spectra. The spectra was standardized to 0.00 ppm with TMSP.

3.14. 1H-NMR recovery

Approximately 1 mg of amygdalin standard was placed into a capped vial with 350 µl of deuterium oxide and 300 µl of TMSP. The ^{1}H-NMR conditions were those described in 3.13. The same procedure was followed for prunasin but using 0.4 mg of standard.

3.15. Moisture, pH and soluble solids measurements

Moisture in freeze-dried kernels and shells was measured with a Denver Instrument I-30 Moisture Analyzer (Bohermia, NY, USA). pH and soluble solids (as °Brix) were measured in the juice with a Denver Instrument pHmeter and an Abbe Refractometer (Leica Auto, Wetzlar, Germany) respectively.

3.16. Results

Results were expressed as mg of cyanogenic glycoside (amygdalin or prunasin)/g fresh or dry material, mg or µg of cyanogenic glycoside (amygdalin or prunasin)/kg or g fresh or dry material (ppm), mg of HCN equivalents/kg of fresh or dry material (ppm). Some results were expressed as µg HCN equivalents/ kernel or pit. To calculate the dry matter, the sample weight was adjusted by the moisture content obtained in 3.15. To obtain the

HCN equivalents, the amygdalin concentration was divided by 16.9260 (equivalent to multiply it by 0.0591). These values correspond to the molar masses ratios, assuming that 1 mol of amygdalin could be converted to 1 mol of HCN. Results for kernels and shells are expressed as mean ± SD of the extraction triplicates of each experimental replicate. The mean from the three experimental replicates was calculated. Results for juice are expressed as mean ± SD of the three different experimental replicates.

3.17. Statistical analyses

A regression analyses was conducted to check the correlation between HPLC and ^{1}H-NMR results. A mixed model was run to analyze the differences in amygdalin concentration among the types of kernel (cultivars and processed blend), setting the sampling date as a random effect. The analysis was followed by post hoc comparison between types with a Tukey's correction. The results obtained for the heat/acid treatment were analyzed by ANOVA. The ANOVA was conducted on the square roots of the responses and was followed by multiple comparisons with Tukey's correction. The statistical analyses were conducted with JMP® 13 Software (Cary, NC, USA).

4. *Results and discussion*

4.1. Amygdalin and prunasin quantification using HPLC equipped with NH_2 column

Previous studies have analyzed the effectiveness of HPLC as a technique for determining amygdalin using C18, and SH-C18 columns (Bolarinwa et al., 2014; Gómez et al., 1998; Savic et al., 2012). Other studies have determined both, amygdalin

and prunasin, using C18 or Hypercarb columns under specific conditions to quantify these compounds simultaneously (Arrázola-Paternina, Dicenta Lopez-Higuera & Grané Teruel, 2015; Berenguer-Navarro et al., 2002). Although the C18 is widely used for HPLC determinations, Berenguer-Navarro et al. (2002) demonstrated that its use may lead to false positive results and distortion of baseline, and they presented the advantages of using a porous graphitic column. In our study, a NH_2 Spherex column was utilized; this column was also effective for simultaneous quantification of amygdalin and prunasin in cherry kernels, presenting effective separation between peaks of the two cyanogenic glycosides (Figure 2). Linear relations between the absorbance and concentrations were obtained for amygdalin and prunasin when using the variables described in 3.9 (R^2=1 and R^2=0.9992, respectively). The method yielded very good recoveries, 1.03 ± 0.06 for amygdalin and 0.99 ± 0.01 for prunasin.

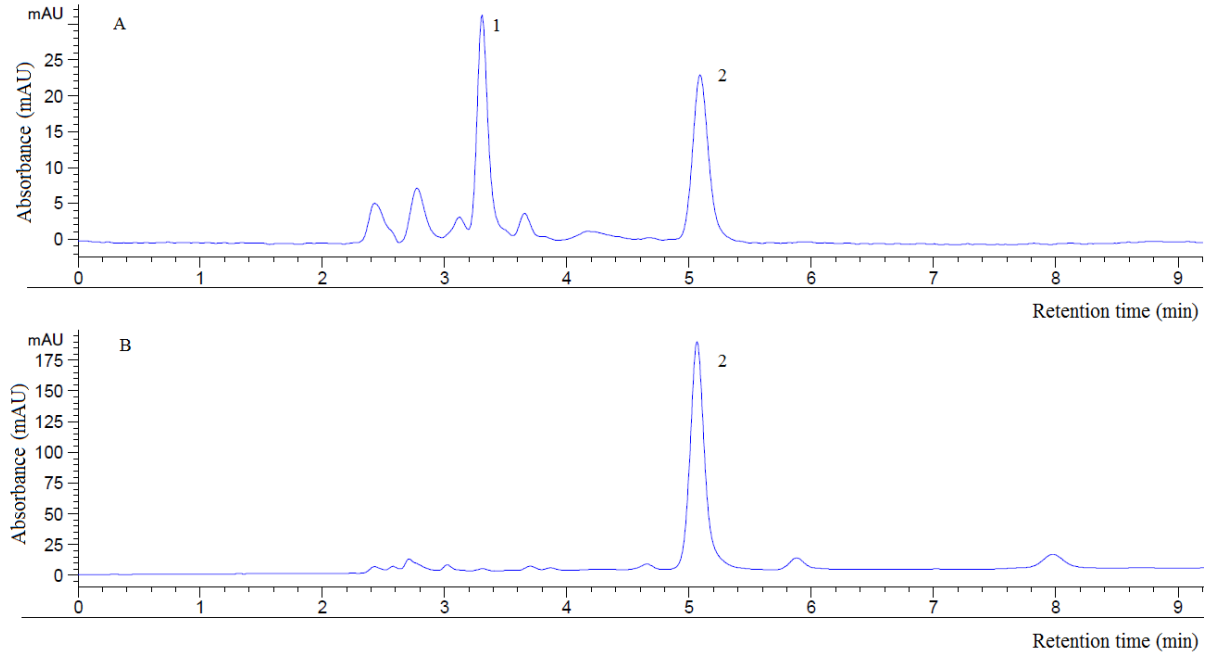

Figure 2: HPLC chromatograms of standards and Balaton cherry kernel. (A) Chromatogram of prunasin, 5ppm (1) and amygdalin, 10 ppm (2). (B) Chromatogram corresponding to diluted Balaton kernel extract. Only amygdalin is observed (2).

A linear relation was also obtained for amygdalin under the conditions described in 3.10 (R^2=0.9991). The HPLC analysis of the cherry pit samples showed that the only cyanogenic glycoside detected in the pits was amygdalin and it was only present in the kernels.

4.2. ^{1}H-NMR for amygdalin and prunasin quantification in different cherry components

^{1}H-NMR has been previously used by Santos Pimenta et al. (2014) to determine amygdalin and prunasin in leaves of *Prunus serotina*. In this study, some of those conditions were adjusted as previously described (3.13) in order to get better resolution in the matrices analyzed. The proton selected for these glycosides quantification was the one located between the benzyl and cyano groups, since it resonates in a non-crowded area of the spectra in both cases (figure 3 and 4).

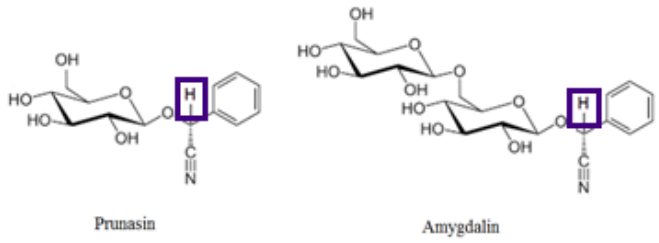

Figure 3: Protons selected in prunasin and amygdalin for quantification by ^{1}H-NMR.

After standardizing the internal standard (TMSP) to 0.00 ppm in the spectra, the amygdalin peak was expected to appear at a frequency between 5.91 and 5.92 ppm and prunasin at 5.93 ppm. Sixty four scans were sufficient to obtain sharp peaks that could be used for quantification in kernels and shells. However, 512 and 1024 scans were necessary in the flesh of the different cherries and in the processed juice respectively to reduce the noise in the baseline and to identify clear peaks that could be integrated.

Results obtained for kernel and shells by ^{1}H-NMR were comparable to those obtained by HPLC. Amygdalin was the only cyano-glycoside identified and, within the pits, it was only present in the kernels. The ^{1}H-NMR also proved to be a convenient and effective way of quantifying amygdalin in cherry flesh and juice due to easy sample preparation and clear identification in the spectra. Recoveries of amygdalin and prunasin were similar to those obtained by HPLC (1.05 ± 0.06 for amygdalin and 1.04 ± 0.01 for prunasin). The amygdalin recovery is comparable to the one obtained by Santos Pimenta et al. (2014). This method provides a practical way of analyzing cyanogenic glycosides without the need of having calibration curves.

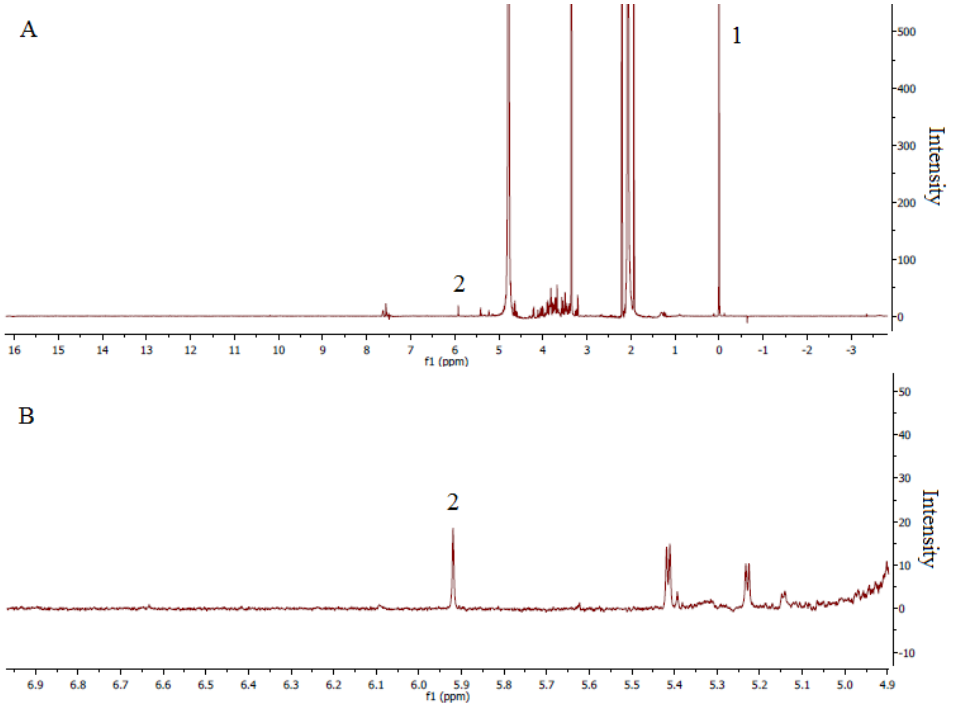

Figure 4: Amplified ^{1}H-NMR spectra of Balaton cherry kernel extract. (A): Internal Standard, TMSP, is represented by peak 1. Amygdalin peak in the extract is represented by peak 2. (B): Amplified spectra in the amygdalin peak area.

4.3. HPLC and ^{1}H-NMR correlation

These two techniques proved to be useful in the quantification of amygdalin in kernels of tart cherries. A strong correlation, represented by the following equation, was found between HPLC and ^{1}H-NMR:

ppm of amygdalin (^{1}H-NMR) = -584.8936 + 1.0243119 * ppm of amygdalin (HPLC)

Figure 5 shows the results obtained by the two methods and their correlation. In the figure, the smallest values correspond to the processed kernels, the intermediate ones to Montmorency and the largest values to the Balaton cultivar. It was observed that the slope is not significantly different from 1 at the 95% confidence level, suggesting that the results of these two methods are in strong linear agreement.

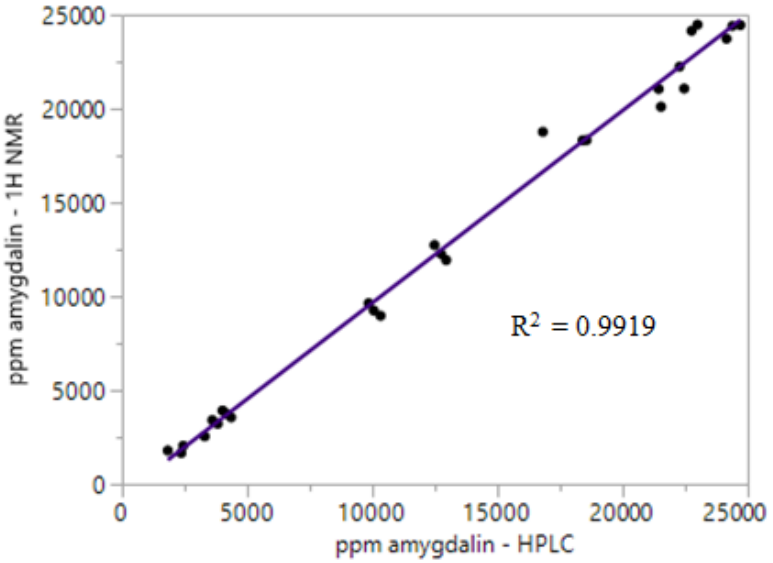

Figure 5: Amygdalin concentration in cherry kernels obtained by HPLC and ^{1}H-NMR (ppm equal to µg amygdalin/g dry kernel or mg amygdalin/kg dry kernel). The correlation between these two methods is represented by the linear relationship.

4.4. Amygdalin and prunasin identification and quantification in unprocessed cherry kernels, shells and flesh.

Amygdalin was the only cyanogenic glycoside detected and it was present in the kernels and flesh of the two cultivars analyzed (Table 1). No amygdalin was detected in the shells. These results are in agreement with previous studies conducted on other products of the Rosaceae family in which it was reported that, during ripening, the prunasin content decreased or was not even detectable (Arrázola-Paternina et al., 2015; Swain et al., 1992). Within the kernels, Balaton was the cultivar that presented the highest concentration of amygdalin, and a significant difference was observed between its mean and the Montmorency's results ($p=0.0085$). Over the three experimental replicates, the average concentration of amygdalin in dry kernels was 23.0 ± 1.3 and 13.6 ± 4.0 mg g^{-1} for Balaton and Montmorency respectively. These values are comparable to results obtained for seeds of other stone fruits as the concentration of amygdalin for plums and apricot kernels published by different authors ranged between 0.44 - 39.70 and 14.37-80.00 mg g^{-1} respectively (Bolarinwa et al., 2014; Gómez et al., 1998; Miao, Zhao, Zhu, Li, & Zhao, 2013; Savic et al., 2012). Specifically, results obtained in this study for tart cherry kernels are higher than those reported by Bolarinwa, et al. (2014) for black and red cherry seeds (2.68 and 3.89 mg g^{-1}). In contrast, Voldrich and Kyzlink (1992), who studied Morello cherry seeds (unknown cultivar), reported a higher value of amygdalin (65 mg g^{-1}) than the ones we observed for the cultivars analyzed. Differences in results may be explained by several factors such as type of cultivar, flowering/ maturation stage, extraction process and quantification method.

Table 1: Amygdalin and HCN equivalents (HCN eq) results for each experimental replicate of different cherry samples (BK=Balaton kernel, MK=Montmorency kernel, PK=Processed kernel, BFJ= Balaton Flesh Juice, MFJ=Montmorency Flesh Juice, PJ= Processed Juice). Kernel results are expressed as mean ± SD of the extraction replicates (n=3). Averages of experimental replicates are expressed as mean ± SD (n=3). For the flesh and processed juices the mean ± SD is calculated on the experimental replicates.

	mg amygdalin/g dry kernel		**mg amygdalin/g kernel (or juice)**		**mg HCN eq/kg dry kernel**		**mg HCN eq/kg kernel (or juice)**		**µg HCN eq/kernel**	
	HPLC	**^{1}H-NMR**	**HPLC**	**^{1}H-NMR**	**HPLC**	**^{1}H-NMR**	**HPLC**	**^{1}H-NMR**	**HPLC**	**^{1}H-NMR**
BK 1	22.1 ± 0.5	21.1 ± 1.1	12.8 ± 0.3	12.2 ± 0.6	1305 ± 29	1249 ± 63	756 ± 17	723 ± 37	68.3 ± 1.5	65.3 ± 3.3
BK 2	24.4 ± 0.3	24.2 ± 0.4	16.8 ± 0.2	16.7 ± 0.3	1442 ± 16	1429 ± 24	995 ± 11	986 ± 17	79.1 ± 0.9	78.4 ± 1.3
BK 3	22.4 ± 0.8	23.2 ± 1.9	15.0 ± 0.6	15.6 ± 1.3	1323 ± 49	1372 ± 112	886 ± 33	919 ± 75	66.2 ± 2.5	68.6 ± 5.6
Avg. Exp. Reps. BK	**23.0 ± 1.3**	**22.9 ± 1.6**	**14.9 ± 2.0**	**14.8 ± 2.3**	**1357 ± 75**	**1350 ± 92**	**879 ± 119**	**876 ± 136**	**71.2 ± 6.9**	**70.8 ± 6.8**
MK 1	10.1 ± 0.2	9.3 ± 0.3	5.6 ± 0.1	5.2 ± 0.2	595 ± 14	549 ± 20	333 ± 8	307±11	25.5 ± 0.6	23.5±0.8
MK 2	12.7 ± 0.2	12.3 ± 0.4	7.6 ± 0.1	7.3 ± 0.2	752 ± 11	727 ± 24	447 ± 8	433±14	29.2 ± 0.5	28.3±0.9
MK 3	17.9 ± 1.0	18.5 ± 0.3	11.8 ± 0.6	12.1 ± 0.2	1059 ± 57	1091 ± 15	695 ± 37	716±10	34.5 ± 1.9	35.5±0.5
Avg. Exp. Reps. MK	**13.6 ± 4.0**	**13.4 ± 4.7**	**8.3 ± 3.1**	**8.2 ± 3.5**	**803 ± 236**	**789 ± 276**	**492 ± 185**	**485 ± 209**	**29.7 ± 4.5**	**29.1±6.1**
PK 1	4.0 ± 0.2	3.6 ± 0.4	2.4 ± 0.1	2.1 ± 0.2	237 ± 11	214 ± 22	140 ± 6	126 ± 13	10.4 ± 0.5	9.4 ± 1.0
PK 2	2.2 ± 0.3	1.8 ± 0.2	1.3 ± 0.2	1.1 ± 0.1	131 ± 20	108 ± 12	77 ± 12	64 ± 7	6.8 ± 1.0	5.7 ± 0.6
PK 3	3.8 ± 0.5	3.2 ± 0.5	2.2 ± 0.3	1.9 ± 0.3	222 ± 32	187 ±3 2	131 ± 19	111 ± 19	12.2 ±1 .8	10.3 ±1 .8
Avg. Exp. Reps. PK	**3.3 ± 1.0**	**2.9 ± 0.9**	**2.0 ± 0.6**	**1.7 ± 0.6**	**196 ± 57**	**170 ± 55**	**116 ± 34**	**100 ± 33**	**9.8 ± 2.7**	**9. 8± 2.7**
BFJ (Avg. Exp. Reps.)	-	-	-	**0.09 ± 0.01**	-	-	-	**5.41 ± 0.26**	-	-
MFJ (Avg. Exp. Reps.)	-	-	-	**0.07 ± 0.01**	-	-	-	**4.00 ± 0.75**	-	-
PJ (Avg. Exp. Reps.)	-	-	-	**0.06 ± 0.01**	-	-	-	**3.52 ± 0.75**	-	-

The impact of having different experimental replicates was also evaluated in the kernels, and significant differences were found within the cherry cultivars depending on the date in which they were sampled. Eighty one percent and 98% of the total variability in Balaton and Montmorency seeds results is explained by the date in which the samples were taken. This may be explained due to differences in the maturation stage when sampled. Although cherries were collected approximately at 70 days after flowering they were not sampled at the same moment since they were collected from different sources. As explained by Swain et. al. (1992), impact of maturation stage on the amount of cyanogenic glycosides is highly significant, so only a few days of difference in the sampling could have been responsible for the differences within cultivars. Amygdalin

and prunasin contents were also evaluated in the flesh of the two different cultivars. The objective was to quantify the initial amount of these compounds in order to later understand the effect of the juicing process in their concentration in different components of the cherries. As in the kernels, amygdalin was the only cyano-glycoside detected in both cultivars and their concentrations were significantly different at $p<0.05$. Amygdalin concentration in the Balaton's flesh juice was 0.09 ± 0.01 mg g^{-1} and 0.07 ± 0.01 mg g^{-1} in the Montmorency's. If transformed to HCN equivalents Balaton and Montmorency flesh juices presented 5.41 ± 0.26 and 4.00 ± 0.75 mg kg^{-1} respectively. These values correspond with previous results published, in which the concentration of HCN in cherry juice ranged between 0.5 to 23 mg HCN/kg or L (EFSA 2004; FAO/WHO, 2012; IPCS, 2004). The variability on the results published may be explained due to differences in processing, amount of pits during the juicing operation and quantification methods.

4.5. *Effect of juicing on the amygdalin content of cherry pits and juice*

The processed pits consisted of a blend of Montmorency and Balaton stones obtained after removing them from an industrial juicing process. Taking this into consideration, the results obtained for processed kernels were compared against each cultivar to compensate for sampling variability. The average amygdalin concentration in the processed kernels was 3.3 ± 1.0 mg g^{-1} (dry basis), and was significantly different from concentrations found in unprocessed kernels of Balaton ($p=0.0002$) and Montmorency ($p=0.0055$). After the juicing process, an 86% and 76% reduction in the amygdalin concentration was observed if compared to results obtained for Balaton and

Montmorency cultivars respectively. Figure 6 displays the amygdalin concentration on the different types of kernels analyzed. Amygdalin was not identified in the processed shells but it was found in the cherry juice obtained from the process (^{o}Brix = 13.87 ± 2.20). The final concentration of this compound in the juice was 0.06 ± 0.01mg g^{-1}, equivalent to 3.52 ± 0.75 mg HCN kg^{-1} (Table 1). This result is in agreement with values published earlier for HCN in cherry juice, as previously stated. However, it is higher than the value reported by Voldrich and Kyzlink for Morello cherries' canned pulp (1.120 mg HCN kg^{-1}). At a significance level of 0.05 the amygdalin concentration obtained in the processed cherry juice is significantly different from the Balaton's flesh juice but not significantly different from the Montmorency's flesh juice. Nevertheless, results of these three different samples are comparable.

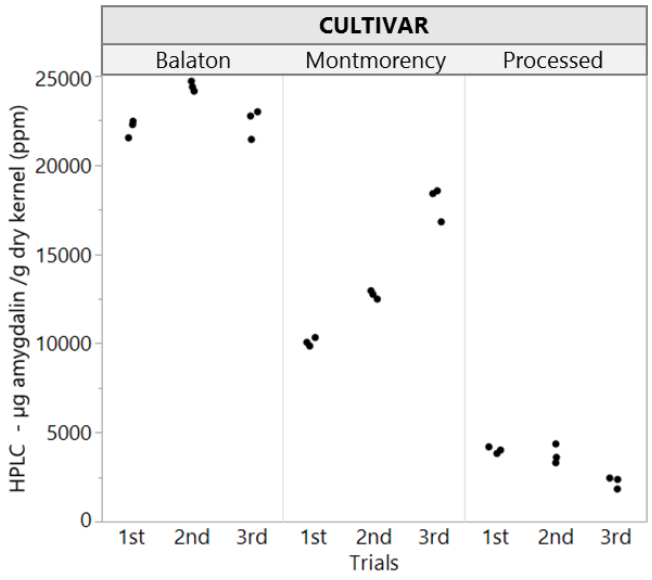

Figure 6: Amygdalin concentration (ppm) in kernels of different cherry cultivars and their processed blend. The trials represent the different experimental replicates. Analytical replicates are represented by the individual points in each trial.

There has been research conducted on the processing effects on cyanogenic glycosides content in food and plants. Boiling has been discussed as a possible removal method due to leaching or hydrolysis of these compounds followed by evaporation of free cyanide (Oke, 1994). However, boiling proved to be inefficient in the total removal of cyanogenic glycosides due to inactivation of the hydrolytic enzymes at higher temperatures (Montagnac et al., 2009; Oke, 1994). Terefe, Sheean, Fernando and Versteeg (2013) showed that inactivation of almond β-glucosidase easily occurs above 60^{o}C being almost completely inactivated in 2 minutes at 80^{o}C. However, they also reported that at 60^{o}C the enzyme had the highest activity. Ketudat Cairns and Esen (2010) also reported similar temperature ranges for inactivation and highest enzymatic activity of β-glucosidase. They reported that these enzymes can be stable between pH 4 and 9. However, Terefe et al., 2013 demonstrated that the thermostability decreased significantly at pH 4 if compared to higher pHs, suggesting that lower values can significantly affect the stability of the enzyme. Considering that the pH in the juice was 3.58 ± 0.05, it can be suggested that if the glucosidase in the seeds had contacted the juice during the operation, their thermostability could have been affected.

Taking into consideration that the amygdalin concentrations in unprocessed flesh and in processed cherry juice were similar, we can first conclude that the reduction on the amygdalin content in the processed kernels is unlikely to be due to solubilization of the compound. Swain et al. (2012) reported the presence of hydrolytic enzymes in black cherry seeds. As a consequence, enzymatic activity may be a possible mechanism by which this reduction could be explained. In this study, cherries were heated up to 79^{o}C. Before reaching the final process temperature, at which the enzyme will be mostly

inactivated, there is a temperature gradient in which the β-glucosidase may have hydrolase activity that could contribute to the reduction of the amygdalin content in the kernels. This is in agreement with Voldrich and Kyzlink (1992) who studied the effect of canning on the HCN content on different stone fruits. They concluded that during heat processing, enzymatic hydrolysis of glycosides plays a major role and that long heating at low temperatures activates hydrolytic enzymes generating HCN as final product.

4.6. Effect of post- process heat/acid treatment in amygdalin content on cherry kernels

A subsequent heat/acid treatment was applied to the processed pits to determine whether a further amygdalin reduction could be achieved in the kernels. The parameters were chosen to mimic the juicing process with some modifications. Citric acid was the only acid source added and it was applied in a higher concentration than that in the regular juice. These variables were set to understand the effect of heat and acid on the amygdalin content under extreme conditions. Citric acid was chosen since it is naturally present in the cherry juice and it is an ingredient commonly used in the food industry. Table 2 shows the amygdalin concentration of samples exposed to the heat/acid conditions for different periods of time and their corresponding control at Time 0. A significant reduction of 31% was obtained after 90 minutes if compared to the control ($p<0.05$). Afterwards, amygdalin concentration remained stable (Figure 7). At 210 minutes a final average reduction of 47% was achieved. However, this value is not significantly different from the result obtained at 90 minutes.

Table 2: Amygdalin concentration and HCN equivalents of cherry pits exposed to a heat acid/ acid treatment at 85ºC with a 2 M citric acid solution for different times. Results are expressed as mean ± SD of the analytical replicates (n=3).

Minutes of processing	mg amygdalin /g dry kernel	mg amygdalin /g fresh kernel	mg HCN eq/kg dry kernel	mg HCN eq/kg fresh kernel	µg HCN eq/ kernel
0	3.03 ± 0.67	1.69 ± 0.38	179 ± 40	100.0 ± 22.2	9.2 ± 2.0
30	2.53 ± 0.20	1.40 ± 0.11	150 ± 12	82.5 ± 6.4	7.6 ± 0.6
60	2.18 ± 0.42	1.22 ± 0.23	129 ± 25	72.3 ± 13.8	6.6 ± 1.3
90	2.08 ± 0.10	1.16 ± 0.05	123 ± 6	68.3 ± 3.2	6.3 ± 0.3
120	2.04 ± 0.07	1.15 ± 0.04	121 ± 4	67.7 ± 2.4	6.2 ± 0.2
150	2.04 ± 0.10	1.15 ± 0.06	120 ± 6	68.0 ± 3.3	6.2 ± 0.3
180	2.12 ± 0.05	1.18 ± 0.03	125 ± 3	70.0 ± 1.8	6.4 ± 0.2
210	1.61 ± 0.22	0.89 ± 0.12	95 ± 13	52.6 ± 7.2	4.8 ± 0.7

In this scenario, it is assumed that there is almost none β-glucosidase activity since, as previously discussed, the pits obtained from the juicing operation were exposed to high temperatures that may have inactivated the enzyme. Since a significant reduction was observed by applying this process we may consider that there might be other mechanisms by which the amygdalin concentration could be reduced in the cherry kernels. Oke (1994) explained that boiling, although inefficient for total removal, may be a possible mechanism for cyanogen reduction due to leaching or hydrolysis. Considering this, solubilization is one of the possible mechanisms by which the amygdalin content decreased in the pits. The other possible mechanism that can explain the amygdalin loss, is the hydrolysis of the molecule into HCN and benzaldehyde, due to acid and heat conditions. Acid hydrolysis has demonstrated to be an effective technique for quantification, in which cyanogenic glycosides are broken by the action of acid and heat, releasing HCN that can be quantified by a colorimetric reaction. Although strong acids, such as sulfuric acid, are usually applied for this procedure, we

can suggest that a less efficient, but similar process may take place during these heat/acid treatment. However, further research monitoring HCN and benzaldehyde content should be conducted to confirm this.

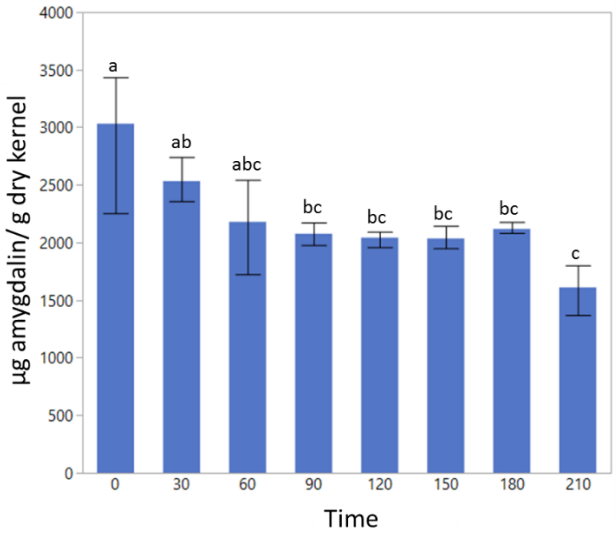

Figure 7: Amygdalin concentration in kernels of processed cherry pits exposed to a heat/acid treatment at 85ºC with a 2 M citric acid solution for different times. The chart represents the mean values and the maximum and minimum results of the analytical replicates (n=3). Levels not connected by the same letter are significantly different at $p<0.05$ (The ANOVA was conducted on the square roots of the responses and was followed by multiple comparisons with Tukey's correction).

4.7. Limits assessment

Although pits of cherries are not intended to be directly consumed by humans, they are a potential source of cyanogens that should be investigated. Balaton and Montmorency kernels presented a significant amount of amygdalin, equivalent to 71.2 ± 6.9 and 29.7 ± 4.5 µg HCN/ kernel or pit. Considering the PMTDI defined by JECFA (FAO/WHO, 2011) for cyanide equivalents (20 µg/kg bw/day) and assuming that all the amygdalin will be converted into HCN due to the presence of glucosidases in the kernels or in the human microflora, a child of 20 kg will reach that limit by only ingesting 6 kernels (or

pits) of Balaton or 14 of Montmorency. However, kernels obtained from the juicing process and from the heat/acid treatment, contained lower levels of HCN equivalents and the probability of reaching the limit is less likely to occur. After the juicing process the concentration of HCN in a whole pit was 36.2 ± 10.4 mg/kg (fresh weight), which is comparable to the limit defined for sweet cassava (50 mg/kg) (FAO/WHO, 2012). Considering that pits are a potential saleable supply, from which new edible products could be made, this value represents a good starting point. If further processing is applied, values comparable to those established for edible cassava products may be reached. In the cherry's flesh and juice values between 3.52 and 5.41 µg HCN equivalents g^{-1} were detected. Considering the PMTDI previously described a child will need to consume between 74 and 114 grams to reach these limits (FAO/WHO, 2011). These values are also above the limits defined by EFSA (2004) for stone fruit products (0.5 – 2 mg CN^{-} kg^{-1}). Although Swain et al. (1992) mentioned that the flesh should be acyanogenic due to the lack of the corresponding enzymes, they also mentioned that if sufficiently chewed they can get in contact with active enzymes in the seed kernels. Thus, it can be stated that although the probability of intoxication by ingestion of flesh and juice is unlikely, amygdalin values present in them cannot be ignored.

5. Conclusions

The HPLC and ^{1}H-NMR methods proved to be useful in the determination of amygdalin and prunasin. Amygdalin was the only cyano-glycoside detected and, within the pits, it was only found in the kernels. A significant reduction in the amygdalin concentration in kernels was observed after the juicing process and post heat/acid treatment. Amygdalin was also found in the cherries' flesh and juice, though its concentration in unlikely to cause poisoning.

6. Acknowledgements

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CHAPTER 2:

EFFECT OF HEAT TREATMENT ON QUALITY, SAFETY AND SENSORY ATTRIBUTES OF CHERRY PIT OIL EXTRACTED BY EXPELLER PRESSING

1. Abstract

Cherry pits, a by-product of cherry processing operations, could turn into a saleable supply if an application is found. This study aimed to evaluate the oil extraction from sour cherry pits by expeller pressing and to assess the impact of heat treatments on yield, sensory and quality attributes. Oil yield ranged from 2.9-4.4% improving with lower moisture contents (7.8-10.2%). Oils did not present significant differences in fatty acids content, peroxide values, color, viscosity, refractive index or moisture and volatiles ($p>0.05$) and amygdalin was not detected in them. However, differences were reported on the aroma of these oils.

A 12-week shelf life study and bioactive compounds determinations were also conducted in the roasted pits oil. Oleic and linoleic were the predominant fatty acids (43.1 and 36.0%) being stable through shelf life. Tocopherols and sterols were also found (1100 and 5913 ppm respectively). At the end of shelf life, quality and sensory parameters remained stable.

2. Introduction

The United States is one of the largest producers of tart cherries (*Prunus cerasus*) (FAOSTAT, 2014). In 2014, FAO ranked its production to be the 5th worldwide being surpassed by the Russian Federation, Ukraine, Turkey and Poland. Within the country, Michigan is the state with the largest production with about 74% of the total amount produced, followed by Utah, Washington, Wisconsin and New York. During 2014 and 2015 an average of 250 million pounds of cherries were produced in the United States and an increase was forecasted for 2016, estimating a total of 309.1 million pounds to be produced in the country (USDA, 2016). From the total sour cherry production in the United States, 99% is currently processed, resulting in a large amount of pits that need to be disposed yearly (AgMRC, 2015). Finding an application for this by-product of the cherry industry is significantly important to processors from an economical and sustainable point of view.

Different applications were proposed over the years to utilize the sour cherry pits coming from the industrial operations. While there is research focused on the health benefits associated with the bioactive compounds present in the kernel extracts, others promote the pits as a potential fuel source (Bak, et al., 2006; Michigan Farmer, 2009). Research has also been conducted on the oil extraction from cherry kernels. Studies on this topic, have focused on the characterization of the oil analyzing its yield, nutritional value and quality attributes. Although authors have reported differences in the percentage and type of fatty acids present in the sour cherry kernel oil, most of them concluded that oleic and linoleic acids are the predominant ones (Popa, Misca, Bordean, Raba, Stef, & Dumbrava, 2011; Korlesky, Stolp, Kodali, Goldschmidt, & Byrdwell,

2016; Kamel & Kakuda, 1992; Górnaś, Rudzińska, Raczyk, Mišina, Soliven, & Segliņa, 2016; Matthäus & Özcan, 2009; Yilmaz & Gökmen, 2013; Özcan, Unver & Arslan, 2015). In addition, some studies investigated the presence of other minor components, identifying bioactive compounds like tocopherols and sterols (Bak et al., 2010; Matthäus & Özcan, 2009; Yilmaz & Gökmen, 2013; Górnas et al., 2016; Korlesky et al., 2016).

Although the cherry pit oil may have an acceptable nutritional value, the presence of toxic cyano-compounds in the kernels should also be considered if the oil is to be consumed. Cyanogenic glycosides are secondary metabolites present in a significant number of fruits and plants, including stone fruits. These compounds can represent a risk for intoxication due to the potential amount of hydrogen cyanide that can be produced when hydrolyzed by enzymes present in the same fruit or in the human microflora (Barceloux, 2009; FAO/WHO, 2009). Amygdalin is a cyano-compound commonly found at later stages of fruit maturation within the *Prunus* genus, including cherries (Arrázola-Paternina, Dicenta Lopez-Higuera & Grané Teruel, 2015; Swain, Li, & Poulton, 1992). Previous research conducted on sour cherry kernels reported significant amounts of this compound (Swain et al., 1992, Voldrich & Kyzlink, 1992), however, none of the research conducted in the cherry pit oil has evaluated amygdalin presence in the final product.

Different extraction methods have been designed over the years to obtain edible oils from plants and crops at an industrial scale. Solvent extraction is known as the most efficient technique to be used in seeds. Pressing machines are commonly used in processing plants, mostly applied for fatty fruits such as palm and olives, or as pre-step

in seeds with high oil content before the solvent extraction. The rise in the organic market is currently demanding the use of methods that are free from solvents. In this context, the use of expellers has become very popular due to easiness of operation and lack of solvents during the process. Though less efficient, this methodology constitutes an easy set up for continuous small operations (Hernandez & Kamal-Eldin, 2013).

Previous research conducted in sour cherry pit oil was mostly focused in determining its nutritional value obtaining the oil by solvent extraction. Yilmaz and Gökmen (2013) analyzed the effect of Supercritical CO_2 extraction on the oil composition and monitored the effect of roasting the kernels on certain bioactive compounds. However, to our knowledge, none of the authors analyzed the sensory characteristics or approximate shelf life of the cherry pit oil obtained, making difficult to understand the possibilities for its commercialization. Moreover, there is little information about pits conditioning before extraction and pressing methods as an alternative way for obtaining the oil. The objective of this study was to analyze the effect of different heat treatments (roasting and dehydration) on the yield and nutritional, quality, and sensory characteristics of tart cherry pit oil obtained by expeller pressing. In order to assess the safety of the oil, the amygdalin content was measured. In addition a 12 week shelf life study was conducted in the roasted pits oil.

3. Materials and methods

3.1. Reagents and standards

Sodium Thiosulphate Pentahydrate, Phenolphthalein, Sodium Dodecyl Sulphate and Potassium Iodide were obtained from VWR (Radnor, PA, USA). Potato Starch for Iodometry was from J.T. Baker (Charleston, SC, USA). Isooctane and Ethanol 95% were from BDH® (Radnor, PA, USA). Glacial Acetic Acid was from Chem-Impex International brand (Wood Dale, IL, USA) and Sodium Hydroxide was obtained from Metrohm USA Inc. (Riverview, FL, USA). Amygdalin Standard (>97%) was obtained from Sigma Aldrich (St. Louis, MO, USA). Methanol, Hexane and Acetonitrile HPLC grade were from Fisher Scientific (Waltham, MA, USA). Almond essence was courtesy of Virginia Dare (Brooklyn, NY, USA).

3.2. Samples

A blend of two tart cherry (*Prunus cerasus*) cultivars, Montmorency and Balaton, was obtained from a cherry juice manufacturing facility located in Northeastern New York after a commercial juicing process. The juicing process consisted on heating up to 79^{o}C the two cherry types in a cooker. Once that temperature was reached, the juice and pits were separated in the juicing step. Pits were stored in sealed high density polyethylene buckets (HDPE) at 5^{o}C until used.

3.3. Heat treatments

Pits obtained in 3.2 were rinsed with abundant water until all the pulp was removed.

Three different treatments were applied to the pits. In all the treatments the objective was to get a moisture between 7 and 11%. The first treatment consisted on roasting the pits (R) in a convection oven (Vulcan®) equipped with 6 trays. A single layer of pits was placed on each tray and roasted in two cycles (190ºC for 7 min and 150ºC for 8 more min). The second treatment consisted on dehydration at 74ºC for 4.5 h (D1). The third treatment was a dehydration at higher temperature, 93ºC, for 3.5 h (D2). The two dehydration processes took place on a batch dehydrator (Sausage Maker brand, add model and city) with a capacity of 20 trays. A single layer of pits was placed in each tray each time. Pits were allowed to cool down and 50% of the total load was coarsely grinded in a hammer mill. Pits were stored in sealed HDPE buckets under refrigerated conditions (5ºC) until used.

3.4. Oil extraction by expeller press

To obtain the oil, the treated pits obtained in 3.3 (R, D1, D2) were individually pressed in an Expeller Press (KOMET; Germany). The oils obtained from this process were named RO, D1O and D2O respectively. The average temperature in the heating device, inlet, cage and outlet of the press were 224, 43, 74 and 129ºC respectively. The oils obtained were centrifuged in 50 ml centrifuge tubes at 6000 rpm for 10 min and then transferred to 187 ml clear glass bottles. Bottles were flushed with nitrogen for 15 s, immediately capped and stored in a box protected from light at room temperature until analyzed.

3.5. Oil quality parameters

The three different oils obtained in 3.4. (RO, D1O and D2O) were analyzed for free fatty acids, peroxide value, moisture and volatiles and refractive index (20^{o}C) following the AOCS official methods (Ca 5a-40, Cd 8b-90, Ca 2c-25, Cc 7-25). Samples were analyzed for color by reading the CIE L*, a*, b* coordinates in a HunterLab, Ultrascan Vis colorimeter (Reston, VA, USA) and for Viscosity utilizing a Brookfield DV-II+ Pro viscosimeter (Middleboro, MA, USA).

3.6. Shelf life study

A 12 week shelf life study was conducted only in the oil obtained from the roasted pits (RO). The bottles were stored at room temperature (25°C) in a box protected from light and samples were analyzed every 4 weeks. Free fatty acids, moisture and volatiles, peroxide index, refractive index and color were analyzed each time following the standards described in 3.5. In each point 3 different unopened oil bottles were analyzed. A sensory evaluation was conducted after 12 weeks (3.8).

3.7. Fatty acids, sterols and tocopherols profiles of the roasted oil sample (RO).

The fatty acids profile, sterols and tocopherols distributions were analyzed by SGS North America Inc. (St. Rose, LA, USA) following Internal Methods or AOCS official procedures. These analyses were only performed in the RO sample for characterization purposes. The analyses were conducted 1 week after the oil was extracted. Fatty acids profile was also analyzed after 12 weeks of shelf life.

3.8. Moisture content in pits

Average moisture contents were calculated for the fresh and treated pits (R, D1 and D2). Ten random samples were taken from each type of pits and were finely cracked with a hammer. Moisture was measured by placing 5 g of sample in a Denver Instrument I-30 Moisture Analyzer (Columbus, OH, USA).

3.9. Sensory analyses

Two sensory analysis were conducted on the color and aroma of the samples. Sixty participants attended each of these studies. For the first sensory study participants were recruited by notifying of the upcoming test to the members of the sensory center database. The first 60 who signed up participated in the test. For the second sensory test, the same 60 people were contacted to participate although some spots were filled by recruiting people as described for the first sensory test.

Each time attendants received a $5 compensation for their participation. Before starting the test, each participant signed a Consent Form in which the risks and benefits were described.

All the data was collected and analyzed in RedJade ® (Redwood Shores, CA, USA).

The sensory studies were conducted following the guidelines and policies of the Cornell Institutional Review Board for Human Participants.

3.9.1. First sensory analysis

The first sensory analysis aimed to assess differences among the three oils obtained (RO, D1O and D2O) and to make a comparison with a commercial almond flavor, which

is similar in the aroma profile. Moreover, RO was characterized by the intensity of different attributes.

3.9.1.1. Color assessment

Two ml of each oil sample (RO, D1O and D2O) were placed in clear glass Petri dishes with lids. The petri dishes were placed in the middle of a paper with black and white background (Figure 1) in a fully illuminated room. Samples were assigned three random digits codes. Participants were asked to rank the samples in order of preference and to answer whether they found the samples "Fairly similar" or "Very different". They were also asked to rate the overall liking of the most liked sample in a 9-point hedonic scale ("Like it extremely" to "Dislike it extremely").

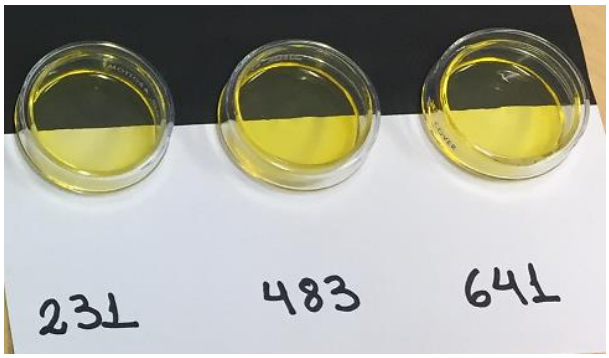

Figure 1: Color assessment of three different cherry pit oils expelled from dehydrated cherry pits at 74°C-4.5 h (231), dehydrated pits at 93°C-3.5 h (641) and roasted pits at 190°C-7 min & 150°C-8 min (483).

3.9.1.2. Aroma discrimination tests

Participants were asked to enter a room with red light and to only evaluate the samples based on the aroma (Figure 2). Three different triangle tests were conducted to understand whether differences between the roasted sample and dehydrated samples, and the roasted sample and an almond extract could be perceived. The samples were

one week old and 1 ml of each oil or 0.5 of almond flavor with yellow colorant were served in opaque polyethylene 5 oz cups with lid labeled with random codes. The triangles were served as follows: RO vs D1O, RO vs D2O, RO vs almond extract. In all the triangles two samples of RO were served. Participants were asked to select the sample that was different and to explain the reason for their decision. To avoid fatigue, it was suggested to them to smell the inside of their elbows between samples.

Figure 2: Sensory cabins illuminated with red light for cherry pit oil aroma evaluation.

3.9.1.3. Aroma attributes test in roasted pit oil (RO)

In order to characterize the aroma of the roasted pit oil an attributes test was conducted. The study took place in the same room that the triangle tests (3.9.1.2.). Participants were first asked to rate the overall liking of the sample on a 9-point hedonic scale ("Like it extremely" to "Dislike it extremely"). Afterwards, they were asked to rate the intensity of the following attributes in a 5 point scale: freshness, fruitiness, sweetness,

caramelized, almond/nutty flavor, cherry fruit aroma, oxidized (old). They were also asked to rate the roasted flavor in a 5 point Just About Right scale.

3.9.2. Second sensory analyses

The second sensory test was conducted 12 weeks after the first one and the objective was to compare the RO freshly produced with the 12-week old sample from the shelf life study (3.6).

For the color assessment the same test described in 3.9.1.1 was conducted. However, in this case the comparison was between a freshly obtained roasted oil sample (RO) and the 12-week old roasted oil (RO12). For the aroma discrimination test only one triangle test was conducted between RO and RO12. The triangle test was conducted following the standards and procedures described in 3.9.1.2., serving two samples of fresh RO. In addition, an attribute test was conducted on RO12. The same attributes rated on 3.9.1.3 were analyzed. The results obtained were then compared to those obtained for the roasted oil sample in the first sensory (3.9.1.3) to understand whether participants perceived differences in the attributes after the 12 weeks of storage.

3.10. Soxhlet

A soxhlet extraction was conducted on the cherry pits to compare the yield obtained with that of the expeller press. Four g of each pit sample (R, D1 and D2) were grinded in a mill at 1725 rpm (General Electric, A-C Motor) placed into cellulose extraction thimbles (Whatman™, 25 mm x 80 mm) and extracted for 6 h with hexane. The solvent was evaporated and afterwards flasks were dried in an oven (IsoTemp Oven,

Fisher Scientific) at 130ºC for 30 min and placed into desiccators. Weight was recorded and continuous 30 min drying intervals were conducted until constant weight. Extractions were performed in triplicates.

3.11. Amygdalin determination in kernel by HPLC

Fresh and treated pits (R, D1, D2) were cracked to obtain the kernels. Kernels were freeze-dried (9 h freezing – 36 h sublimation) in a Harvest Right Freeze-Drier (North Salt Lake, UT, USA) and stored in hermetically sealed glass jars until used. For amygdalin determination, kernels were crushed with a mortar and pestle and 0.7 g were weighed into a cellulose extraction thimble (Whatman™, 25 mm x 80 mm) and extracted with 100 ml of methanol in a Soxhlet apparatus at 65ºC (Gómez, Burgos, Soriano, & Marín, 1998). Solvent was evaporated and the extracts were kept in desiccators overnight. Three extractions were conducted for each sample. Kernel extracts were diluted to 10 ml with a solvent mixture of acetonitrile and water (75:25, v/v) and sonicated in a Branson 2200 equipment for 5 min. A further 1/10 dilution was made. Samples were passed through a 0.45 µm Nylon Syringe filter before injection in the HPLC. An Agilent 1100 HPLC equipment with auto sampler, degasser, quaternary pump and Diode Array Detector set at 210 nm was used. The column was a Phenomenex Spherex NH_2 (250 x 4.60 mm, 5 µm) and it was set at 20ºC. Twenty µl of sample were injected each time and eluted under isocratic conditions at 1 ml/min flow rate with a solvent mixture of acetonitrile and water (75:25, v/v) as mobile phase. Chromatograms were analyzed with Agilent ChemStation Software (Rev. B. 04.03 (16)) by manually

integrating peaks. The HPLC variables were chosen based on the method described by Savic, Nikolic, Savic, Nikolic &, Stankovic (2012).

3.12. Amygdalin determination in cherry pit oil

2 ml of each different oil (RO, D1O, D2O) were placed in centrifuge tubes. Aliquots of 3 ml of methanol were combined with the oil and mixed in a vortex mixer for 1 minute. The tubes were allowed to stand for 1 min to promote phase separation. The methanol supernatant was transferred to another centrifuge tube. This procedure was repeated 4 more times combining all the methanol fractions in the same tube. To evaporate the methanol, the tubes were placed in a nitrogen blow dry station equipped with a water bath at 37^{o}C. The nitrogen pressure was set at 15 psi (103.4 kPa). The dried extract was then reconstituted with 2 ml of a solvent mixture (acetonitrile 85: water 15, v/v) and sonicated for 1 min. The extractions were conducted in triplicate. Samples were passed through a 0.45 µm Nylon Syringe filter before injection in the HPLC. The equipment, column and software used were those described in 3.11. Twenty µl of sample were injected each time and eluted under isocratic conditions with a 1.2 ml/min flow rate. The mobile phase was a combination of acetonitrile and water (85:15, v/v) and the column temperature was set at 30^{o}C. Chromatograms were analyzed with Agilent ChemStation Software (Rev. B. 04.03 (16)) and peaks were manually integrated.

3.13. Amygdalin determination in press cake

The press cake obtained from each different oil extraction was also analyzed. For the extraction, the procedure described in 3.11 was applied. The extract obtained was

diluted to 10 ml using a mixture of acetonitrile and water (85:15, v/v). Samples were filtered through a 0.45 µm Nylon Syringe filter and afterwards injected in the HPLC. The HPLC conditions used were those described in 3.12.

3.14. Calibration Curves for HPLC

Stock solutions of 1000 ppm (µg/ml) of amygdalin in each of the mobile phases were prepared and further dilutions of 10, 20, 40, 60, 80 and 100 µg/ml were made. These solutions were run in the HPLC following the procedures in 3.11 and 3.12.

3.15. HPLC recovery

A recovery test was conducted for the amygdalin extraction in the oil. Approximately 0.0020 g of amygdalin were placed in a centrifuge tube with 2 ml of the roasted oil and mixed in a vortex mixer for 1 min. To analyze the amygdalin recovery, the procedure described in 3.12 was applied. Results obtained were compared to the initial amount of amygdalin spiked.

3.16. Yield and conversion rates

The yield was calculated for the three different oils obtained on a dry basis using the following equation:

%Yield = g of centrifuged oil/ 100 g dry pits

To obtain the dry pits weights, the moisture content in the pits was adjusted with the results obtained in 3.8.

The conversion rate was calculated as g of centrifuged oil/ kg of conditioned pits (pits exposed to heat treatment).

3.17. Capacity measurement

The capacity was measured for all the equipment considering the different treatments applied. Capacity was expressed as kg of pits processed in 1 h.

3.18. Statistical analyses

Non-parametric Wilcoxon Tests between pairs were performed to analyze differences among the quality attributes in the oil samples. A one way ANOVA was also conducted on shelf life results to analyze the impact of time on the different parameters evaluated. The analysis was followed by post-hoc comparison between types with a Tukey's correction. The sensory discrimination tests and aroma attributes were analyzed using the Chi Square test. The statistical analyses was conducted in JMP® 13 Software (Cary, NC, USA).

4. Results and discussion

4.1. Yield and capacity

The average moisture in the fresh pits obtained from the juicing process was 35.25 ± 0.74%. In order to obtain an acceptable yield, the roasting and dehydration treatments were designed to get an average moisture in the pits between 7 and 11%. This range was selected based on the study by Adejumo, Inaede and Adamu (2013), in which they

reported that the highest yield for *moringa oleifera* seeds was obtained with moisture contents between 7.28 and 10% and that yield decreased at 15% moisture. Gunstone (1998) stated that for good crushing and storage of sunflower seeds, moisture levels should be reduced to 10%, and even 8% if stored for longer times. After the roasting process pits' moisture was 10.24% while in D1 and D2 the final moisture contents were 8.64±0.25 and 7.84±0.39% respectively. The yield obtained was inversely proportional to the moisture content (Table 1). Pits obtained in D2 had the lowest moisture but the highest yield while the roasted pits had the highest moisture and lowest yield. Adejumo et al. (2013), suggested that the yield of *moringa* seeds decreases above 10%. In this study a significant reduction on the oil's yield was observed between 7.84 and 8.64%. At 10.24% the reduction was even more significant. This suggests the optimal extraction moisture for sour cherry pits may be at lower values.

The maximum capacity for each process was measured. Figure 3 displays a flowchart of the conditioning and extraction processes with their corresponding capacity. For conditioning treatments, capacity was expressed as total kg of pits that could be processed (feed) and the amount of product that could be obtained (output) in one working shift of 12 h considering loading and unloading times.

Table 1: Effect of dehydration and roasting on moisture content of cherry pits and oil yield

Treatment	Temperature-Time	Moisture (% w/w)	Yield (% w/w)
Low temperature dehydration (D1)	74°C-4.5 h	8.64	3.44
High temperature dehydration (D2)	93°C-3.5 h	7.84	4.44
Roasting (R)	190°C-7 min & 150 °C-8 min	10.24	2.87

These units were chosen to allow comparisons among the different conditioning treatments which were batch operations with different processing times. The roasting procedure presented the highest capacity with a total volume of 94.5 kg that could be processed in a 12 h shift. This was followed by D2 with 51.0 kg and finally the D1 treatment with 34.0 kg in 12 hours. The output of each of these treatments, expressed as kg of conditioned pits in 12 h, were 27.4 for R, 11.3 for D2 and 8.3 for D1. In contrast to the conditioning treatments, the expeller press operated in continuous flow. The conversion rates (g of oil produced per kg of conditioned pits) were 25.7 for R, 40.9 for D2 and 31.4 for D1 and on average the press was able to process 4.1 kg of conditioned pits in 1 h. Considering these factors, and the output that can be obtained from each conditioning process, although the R treatment had the lowest conversion in one working shift of 12 h it will produce 706.4 g of oil while D2 and D1 will produce 464.2 and 261.9 g of oil respectively. Moreover, the roasting procedure allows the process to be operated almost continuously since there are pits available to process every 15 min. Although the R process had the lowest yield under these conditions it was the process that provided the largest amount of oil in one working day. If the process is to be scaled up, the moisture content should be adjusted to obtain most of the oil present in the pits, which will make the operation even more efficient. Considering its fastest extraction, the roasted pit oil was selected for the shelf life study and sensory evaluation.

A Soxhlet extraction was conducted only in the roasted pits to analyze the differences in the extraction's yield. While 2.87% yield was obtained by expeller press, an average of 4.45% was obtained by Soxhlet concluding that the solvent extraction is more efficient for oil extraction under these conditions. Although this result was expected,

since solvent extraction is reported to be the most efficient method (Hernandez & Kamal-Eldin, 2013), the yield measured is similar to that obtained for D2 (4.44%). This suggests, as previously explained, that if the moisture content is optimized, better yield could be achieved. The values obtained either by expeller pressing or by Soxhlet are lower than those reported by Farrohi and Mehran (1975) who reported a yield range between 5.8 and 7.7% for various sour cherry pits. Differences between the results may not only be due to cultivar variations but also to the way in which yields were calculated. While in this study yield was directly measured from the operation, Farrohi and Mehran (1975) calculated it by extracting the oil from the kernel and then reporting the result on the whole stone.

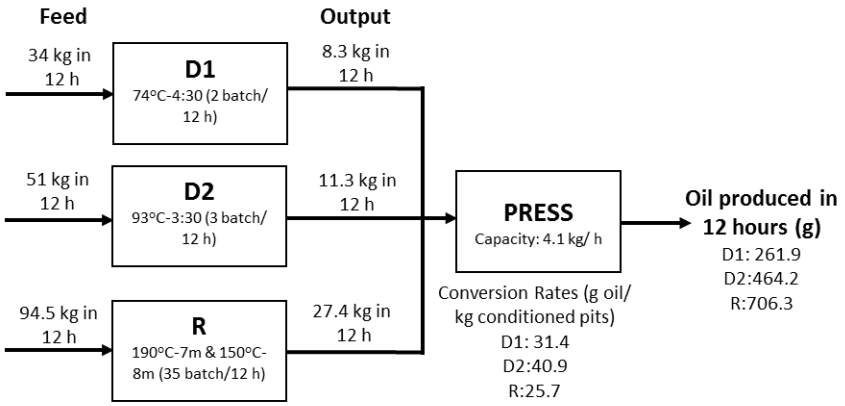

Figure 3: Flowchart of cherry pit oil extraction process.

4.2. Color (CIE L a* b*), refraction index and viscosity in cherry pit oils*

The color of the different oils was characterized using the CIE L* a* b* scale. In this scale three different values are obtained, L*, a* and b*. L* is an estimate of how light or dark the sample utilizing a scale that ranges from 0 (black) to 100 (white). In the "a*" scale (-100 to +100), the red colors are represented by positive numbers while green ones by the negative numbers. Finally in the "b*" scale, which also ranges from -100 to

+100, the blue is represented with negative numbers and yellow with positive ones. The L*, a* and b* values obtained for the different samples are reported in Table 2 and they ranged from 65.50 – 67.75 for L*, 12.36-14.01 for a* and 71.16-74.64 for b*. On average, results are comparable to those obtained for roasted sesame seed oil (L*=71.1, a*=9.4, b*=53.8) (Seol, Jang, Kim, & Lee, 2012), being the cherry pit oil slightly darker, more red and yellow. The color differs more from that of the pumpkin seed oil (L*= 44.8, a*= - 0.18, b*= 28.88) by being lighter and having more red and yellow components (Rezig, Chouaibi, Msaada, & Hamdi, 2012). Although no significant differences were found ($p>0.05$) some trends were observed. In all the cases results were in the light side for L* and in the yellow part for b* but D1O presented the highest values for these two parameters while RO presented the lowest values. For the a* scale the RO sample presented the highest value and D1O the lowest, being the red color more intense in the roasted samples. Park, Seol, Chang, Yoon and Lee (2011) explained that the darkening in perilla oil samples when exposed to higher roasting temperatures or times was due to the occurrence of different chemical reactions, such as Maillard or caramelization, in the seeds. Considering that roasted pits were exposed to the highest temperatures and a darker color was obtained in that oil we can suggest that similar reactions may have occurred in it and that there might be a correlation between the different color components and the temperature at which the pits were processed.

The Refractive Index at 20^{o}C was also measured in the oils. The refractive index is characteristic for each different oil since it is associated with molecular weight, fatty acids chain length and degree of unsaturation and conjugation (Gunstone, 2002). The range of refractive indexes obtained for the oils was between 1.47676 and 1.47725,

comparable to the one reported by Özcan et al. (2015) for sour cherry pit oil (1.4702 at 20°C). Farrohi and Mehran (1975) reported a range between 1.4692 and 1.4721 (40°C) for sweet and sour cherries. The oils had similar viscosities with no significant differences ($p>0.05$). Viscosity is a parameter highly dependent on the temperature, thus any comparison must be done under similar conditions. At 25°C, the cherry pit oils had higher viscosities (Table 2) than those reported for soybean, corn and rapeseed oils ranging from 52.3 to 78.8 cP. However, the viscosities were lower than the one obtained in Lesquerella oil at the same temperature (275 cP) (Noureddini, Teoh & Clements, 1992).

Table 2: Quality parameters measured in different cherry pit oils. Results are expressed as mean ± SD (n=3). D1O= Cherry pit oil expelled from pits dehydrated at 74°C-4.5 h. D2O= Cherry pit oil expelled from dehydrated pits at 93°C-3.5 h. RO= Cherry pit oil expelled from roasted pits 190°C- 7 min and 150°C-8 min.

Parameters	D1O	D2O	RO
Refraction Index (20°C)	1.47676	1.47765	1.47725
Viscosity (25°C) (cP)	127.3 ± 0.4	125.0 ± 0.4	127.5 ± 0.3
L*	67.75 ± 0.02	67.11 ± 0.02	65.50 ± 0.04
a*	12.36 ± 0.01	13.07 ± 0.03	14.01 ± 0.02
b*	74.64 ± 0.13	73.89 ± 0.17	71.16 ± 0.02
Free Fatty Acids (% Oleic Acid)	0.53 ± 0.00	0.49 ± 0.02	0.46 ± 0.02
Peroxide Value (meq peroxide/kg)	1.36 ± 0.04	1.17 ± 0.03	0.99 ± 0.02
Moisture and Volatiles (%)	0.48 ± 0.02	0.46 ± 0.00	0.38 ± 0.02

4.3. Free fatty acids, peroxide value and percentage of moisture and volatiles

All the values obtained for free fatty acids were expressed as % oleic acid and ranged from 0.46 to 0.53%. These results were similar to those published by Popa et al. (2011) who reported an acid value on the Montmorency kernel oil of 1 mg KOH/g, which is equivalent to 0.5% Oleic Acid. The values obtained are comparable to the results

reported by authors for cherry kernel oils. Kamel and Kakuda (1992) reported 0.38% and Korlesky et al. (2016) published and acid value 1.45 mg KOH/g, equivalent to 0.73% Oleic Acid. However, Özcan et al. (2015) found higher values for the cherry pit oil at 2.5% Oleic Acid.

The peroxide value is utilized to measure lipid oxidation since peroxides are generally associated with undesirable flavors and reactions in the oil. In the different oils obtained peroxide values ranged between 0.99 and 1.36 meq O_2 kg^{-1}, which were lower than the ones reported by other authors at 1.6 and 2.8 meq O_2 kg^{-1} (Popa et al., 2011; Özcan et al., 2015). Peroxides formation depend on different variables such as moisture content, freshness of the material to be extracted or the oil, storage conditions, and degree of unsaturation, among others. Although no significant differences were observed among the samples, the small differences found may be due to some of the factors previously described alone or in combination.

The moisture and volatiles percentage obtained for the different oil samples was between 0.3657 and 0.4775%. While no significant differences were observed among the samples ($p>0.05$), RO, which was the product exposed to the highest temperature, presented the smallest moisture and volatiles percentage. Comparable results were published by Potočnik and Košir (2017) who explained that at high temperatures reactions such as Maillard occur, and demonstrated that the some aldehydes and alcohols with high volatility are lost after 110 or 150^{o}C in pumpkin seed oil.

4.4. Amygdalin content in pits, oil and press cake

Linear relationships were obtained between the amygdalin concentration and the absorbance areas in the HPLC under the conditions described in 3.11 and 3.12 (R^2 = 0.9999 and R^2 = 0.9998, respectively). The liquid-liquid amygdalin extraction with methanol applied to the cherry pit oils showed a good recovery (1.05±0.02). Table 3 shows the concentration of amygdalin in the kernels, oils and in the press cakes.

The values obtained for amygdalin in the different cherry kernels are comparable to those reported by Bolarinwa et al. (2014) for Black and Red cherry seeds (2.68 and 3.89 mg g^{-1}). However, significant differences were found among all the different kernels. The amygdalin concentration in seeds of stone fruits presents intrinsic variations due to different factors such as cultivar, maturation stage or extraction method. The cherry pits used in this study were a blend of two different sour cherry cultivars (Montmorency and Balaton) obtained after an industrial juicing process. The ratio of these two cultivars could have varied among the samples taken affecting the final amygdalin concentration quantified and causing variability. Swain et al. (1992) demonstrated the impact of the flowering/ maturation stage on the amygdalin content which could have been another reason for variation among samples. Considering this, differences in amygdalin concentration in kernels cannot simply be explained by the heat treatment since other variables must be controlled. In consequence, in this case this measurement was just conducted in order to understand qualitatively whether amygdalin was present in the kernels before the pressing stage. Although amygdalin concentration was significant in all the kernels (fresh and treated), no amygdalin was detected in the oils. However, this cyano-compound was detected in the different press cakes. Variations in results may be

explained by the factors previously mentioned for the kernel. Moreover, the variability could also be explained by the fact that the press cake is a heterogeneous mass of kernel and shell residues which makes it difficult to sample each compound at the same ratio each time. Although Kamel and Kakuda (1992) raised the concern for cyanogenic glycosides presence in stone fruit oil, the authors did not perform the oil analysis in their study. Hosseini, Heydari and Alimoradi (2015) reported values of 0.047 and 0.092 mg g^{-1} for sweet and bitter almond oils however no bibliography was found on amygdalin content in sour cherry pit oil. The solvent free expeller press extraction under the three conditions evaluated demonstrated to be an effective way of extracting the oil without detecting amygdalin in the final product.

Table 3: Amygdalin concentration in cherry kernels, cherry pit oils and press cakes. Results are presented as mean ± SD (n=3).

	Amygdalin Concentration		
Treatment	**Kernels (µg/g dry matter)**	**Oil (µg/ml)**	**Press cake (µg/g dry matter)**
Fresh (control)	4479 ± 295	-	-
Dehydration at low temperature (D1)	1497 ± 132	ND	280 ± 8
Dehydration at high temperature (D2)	3293 ± 215	ND	90 ± 19
Roasting (R)	6865 ± 423	ND	1905 ± 84

4.5. Sensory evaluation

4.5.1. Color evaluation in RO, D1O and D2O.

RO was the most preferred sample as 50% of the participants ranked it in first place when evaluating the oils for color. D2O and D1O were ranked first by 28 and 22% of the participants respectively. Although results indicated RO was the preferred sample, 87% of the participants stated that all the samples were “fairly similar” and within that group 62% liked the samples and 38% “neither liked it nor disliked it”.

4.5.2. Aroma discrimination tests

In the first triangle test (RO vs. D1O), 52% of the participants answered correctly, identifying D1O as the different sample. In the second triangle test (RO and D2O), 68% perceived the differences between the samples. In the last test in which RO was evaluated against an almond flavor, 93% answered correctly specifically mentioning that the almond flavor had an artificial aroma that was “more chemical” and “sharper". In all the cases the Chi Square test demonstrated that samples were significantly different ($p<0.01$), concluding that people can identify the differences among the samples tested.

4.5.3. Characterization of roasted cherry pit oil (RO) and shelf life impact on sensory attributes

4.5.3.1. Attributes Rating

In the first sensory evaluation the aroma of RO was evaluated by measuring its overall liking. After 12 weeks the same study was conducted to evaluate differences.

In the evaluation made with freshly pressed RO, 53% of the participants mentioned that they liked the aroma of the oil, 39% neither liked it nor disliked it and only 8% did not like it. When the same study was conducted with RO12, 56% mentioned they liked the sample, 37% neither liked it nor disliked it and only 7% disliked it (Figure 4). The Chi square test revealed that the Overall Liking results obtained after 12 weeks are not significantly different from results at Time 0 at a significance level of 0.05. An attributes test was also conducted in RO and RO12 to identify clear descriptors and their intensities and to understand the impact of storage time. Results are summarized in

Table 4. More than 50% stated that both samples, RO and RO12 were perceived as very or moderately “fresh” (top two intensities). Even the sample stored for 12 weeks was rated as more “fresher” than the original sample. This result is in agreement with the values obtained for the oxidation perception as 67% expressed that the fresh sample was “not at all” or “not very” oxidized, and after 12 weeks people perceived that samples were even less oxidized (81% ranked the attribute in the bottom two intensities). However, the small changes observed in these parameters after 12 weeks of storage are not significantly different at a 0.05 significance level.

The most intense descriptor was the “Almondy/Nutty” aroma as 88% of the participants placed this attribute in the top two intensities in the fresh sample analysis. Although after 12 weeks its intensity decreased, still 75% ranked it in the top 2 intensities, being the predominant aroma attribute in the cherry pit oil. The second most intense attribute was the sweetness which was ranked in the top two intensities by 63% of the participants in the fresh sample and by 67% in the 12-week old sample. As pits were roasted before to the oil extraction, it was important to evaluate the caramelization or Maillard like aroma. Caramel aroma was mostly ranked in the top 2 intensities for RO and RO12 (43 and 40%, respectively). Although some differences were observed after 12 weeks of storage, for these three attributes (Almondy, Sweet and Caramelized) the changes were not significantly different ($p > 0.05$).

In the “fruity” and “cherry fruit” aroma the same pattern was observed. These two attributes were not very intense in the fresh sample but about 60% more of the panelist ranked RO12 as moderately or very “fruity” and “cherry fruit”. At a significance level

of 0.05, the cherry fruit aroma was the only attribute that increased its intensity significantly after 12 weeks of storage.

To assess whether the roasted aroma in the oil was perceived as right, the roasting level was evaluated. Thirty nine percent of the panelists stated that the roasted aroma in RO was “just about right” and 42% stated that it was “not quite” or “not at all” roasted. In RO12 the percentage of testers who stated that the roasting level was “just about right” decreased (32%) while panelists who stated that roasting level was not enough increased (51%). The changes observed were not significantly different ($p>0.05$).

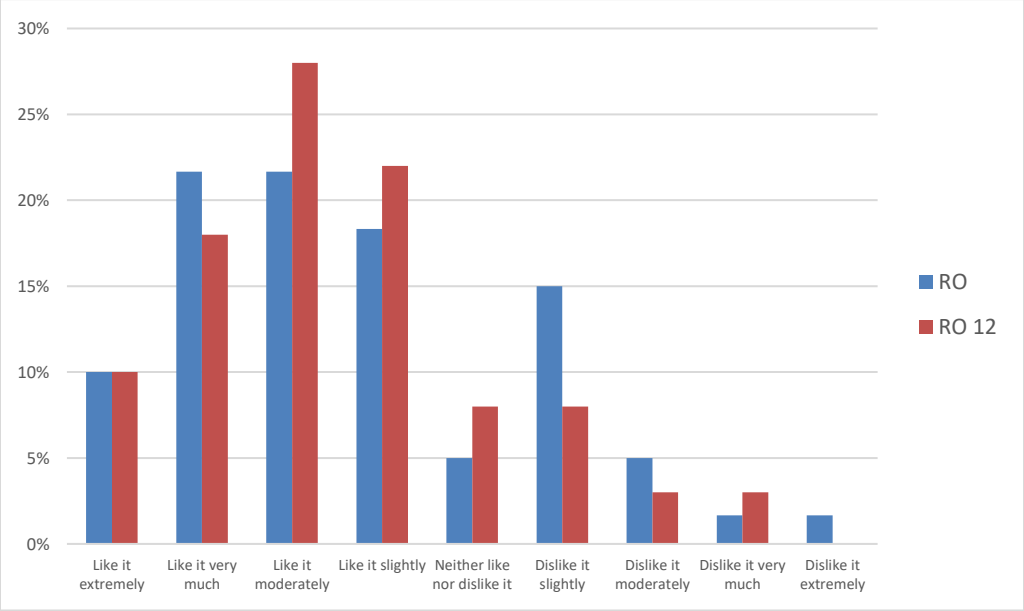

Figure 4: Aroma Overall Liking for roasted cherry pit oil at time 0 (RO) and after 12 weeks of storage (RO 12). Results are expressed as percentage of participants (n=60).

Table 4: Attribute intensity of the roasted cherry pit oil at time 0 (RO) and after 12 weeks of storage (RO12). Results are expressed as percentage of panelists who ranked each attribute for each intensity (n=60). Top 2= Includes percentages corresponding to "Very" and "Moderately" intense. Bottom 2= Includes percentages corresponding to "Not very" or "Not at all" intense.

	Attribute																
	Fresh		**Oxidized**		**Almond/Nutty**		**Sweet**		**Caramelized**		**Cherry fruit**		**Fruity**		**Roasted**		
Intensity	RO	RO12	RO	RO12	RO	RO12	RO	RO12	RO	RO12	RO	RO12	RO	RO12	**Intensity**	RO	RO12
Very	23%	20%	3%	2%	62%	53%	28%	40%	8%	8%	7%	22%	5%	10%	Much too	0%	0%
Moderately	28%	35%	10%	7%	27%	22%	35%	27%	35%	32%	22%	23%	23%	38%	Slightly too	19%	17%
Top 2	**52%**	**55%**	**13%**	**9%**	**88%**	**75%**	**63%**	**67%**	**43%**	**40%**	**28%**	**45%**	**28%**	**48%**	**Top 2**	**19%**	**17%**
Slightly	23%	27%	20%	10%	10%	20%	25%	20%	28%	33%	32%	28%	32%	33%	JAR	39%	32%
Bottom 2	**25%**	**18%**	**67%**	**81%**	**2%**	**5%**	**12%**	**13%**	**28%**	**26%**	**40%**	**27%**	**40%**	**18%**	**Bottom 2**	**42%**	**51%**
Not very	22%	13%	22%	38%	2%	3%	10%	10%	22%	18%	30%	22%	32%	13%	Not quite	20%	29%
Not at all	3%	5%	45%	43%	0%	2%	2%	3%	7%	8%	10%	5%	8%	5%	Not at all	22%	22%

4.5.3.2. Color evaluation

During the second sensory study a color preference test between RO and RO12 was conducted. No significant differences were found in the color of these two samples ($p>0.05$) (52% preferred RO12 and 48% RO). In addition, 100% of the participants mentioned that the samples were "fairly similar", 63% mentioned they liked the sample and the remaining 37% that they neither liked it nor disliked it. When evaluating the oil sample after 12 weeks of storage the panelists could not perceive differences in color compared to the fresh sample and still liked the sample.

4.5.3.3. Aroma discrimination test

During the second sensory evaluation a discrimination test was also carried out between RO and RO12. Fifty seven percent of the participants correctly identified the RO12 as the different sample, demonstrating that the two samples were significantly different ($p<0.001$). Among people who responded correctly, the most common reason for their selection was that the aroma in RO12 was not as strong or sharp as it was in the fresh

sample. Considering all the results of the attributes and discrimination tests, it can be concluded that after 12 weeks of storage, participants were able to identify differences in the aroma of the samples but they still like its overall aroma in the same way or even slightly more.

4.6. Shelf life study in roasted cherry pit oil

RO was chosen for the shelf life analysis due to simplified processing and satisfactory sensory results. Table 5 shows the evolution of the different parameters evaluated through the shelf life. With regards to the color, only slight differences were observed. The light component (L*) gradually decreased through the shelf life, getting darker over time. No specific trend was observed in the red component (a*). The yellow component (b*) significantly decreased until week 8 and then it remained stable for the last 4 weeks. When comparing the instrumental color values with the results from the sensory evaluation, it is clear that the instrument is able to measure differences in the color components but these variations are not perceived by the human eye.

The free fatty acids, expressed as % oleic acid, remained relatively stable throughout the entire shelf life study. Only a significant reduction was observed at week 12 ($p<0.05$). The results are in agreement with those of Crapiste, Brevedan, and Carelli (1999), who reported that % oleic acid was relatively constant through the shelf life in sunflower oil. They stated that a constant value in the free fatty acids may be due to the lack of hydrolytic activity. Through the entire shelf life results were still within the range of results published by other authors (0.38-2.25%) (Kamel and Kakuda, 1992; Popa et. al., 2011; Korlesky et al., 2016).

Peroxide values also remained relatively stable until week 8 but a significant increase on this parameter was observed at week 12 ($p<0.05$). Kozac and Samotyja (2013) analyzed the shelf life of rapeseed oil under different conditions, reporting that peroxide values of the rapeseed oil, stored with different concentrations of oxygen and nitrogen, increased after 40 days at 50ºC (increase was slower with higher nitrogen content). In pressed sunflower oil stored with nitrogen at 47ºC (accelerated study), Crapiste, Brevedan and Carelli (1999) mentioned that there were no significant changes in the peroxide content in the 60 days of shelf life. However, from results reported in their study a slight increase in peroxides content was observed at day 30 followed by a reduction at day 60. In these two studies the maximum peroxide value was observed earlier than in our samples in which we still see an increase at week 12. This can be explained by the fact that those studies were conducted at higher temperatures (accelerated) and ours was conducted at room temperature. Hydroperoxides have no flavor, however they undergo continuous degradation producing secondary oxidation products such as aldehydes, alcohols, ketones and acids among others, which are generally associated with unacceptable odors and flavors. In the sensory attributes test conducted in RO12, 80% of the participants stated that the aroma of the samples was not oxidized, suggesting that the secondary products of this reaction were not yet formed or were present in very low concentration. It is also important to mention that even after 12 weeks the peroxide values were still lower than results reported by Popa et al. (2011) at 1.6 meq O_2 kg^{-1}, and Özcan et al. (2015) at 2.8 meq O_2 kg^{-1} for sour cherry kernel oil. The percentage of moisture and volatiles also remained relatively stable, although a reduction compared to the control was observed at week 12, in agreement with results

obtained in the sensory analysis where RO12 was perceived to have a less intense or less sharp aroma. Overall, after 12 weeks of storage with nitrogen in the headspace, the oil quality parameters remained relatively stable.

Table 5: Quality parameters of roasted cherry pit oil evaluated through the shelf life. Results are expressed as mean ± SD (n=3). Levels not connected by the same letter are significantly different ($p<0.05$).

	Weeks at 25°C			
Parameter	**0**	**4**	**8**	**12**
Refraction Index (20ºC)	1.47725	1.47782	1.47791	1.47768
L*	65.50 ± 0.04 [a]	65.24 ± 0.04 [b]	64.82 ± 0.09 [c]	64.78 ± 0.01 [c]
a*	14.01 ± 0.02 [b]	13.77 ± 0.02 [c]	14.39 ± 0.13 [a]	14.51 ± 0.10 [a]
b*	71.16 ± 0.02 [a]	70.85 ± 0.13 [b]	69.72 ± 0.14 [c]	70.00 ± 0.12 [c]
Free Fatty Acids (% Oleic Acid)	0.46 ± 0.02 [a]	0.47 ± 0.02 [a]	0.43 ± 0.01 [ab]	0.42 ± 0.01 [b]
Peroxide Value (mEq peroxides/kg)	0.99 ± 0.02 [b]	0.99 ± 0.05 [b]	1.08 ± 0.06 [b]	1.32 ± 0.03 [a]
Moisture and Volatiles (%)	0.38 ± 0.02 [a]	0.37 ± 0.01 [ab]	0.38 ± 0.01 [a]	0.33 ± 0.01 [b]

4.7. Fatty acids, tocopherols and sterols content in oil obtained from roasted pits

The fatty acid distribution of the oil obtained from roasted sour cherry pits is displayed on Table 6. Results obtained at time 0 and after 12 weeks of shelf life are not significantly different ($p>0.05$). The two main fatty acids in the oil were oleic and linoleic (43 and 36% respectively). Other authors have also reported these two fatty acids to be the predominant ones in the sour cherry kernel oil. The fatty acids distribution obtained is in agreement with results published by Korlesky et al. (2016), Popa et al. (2011) and Yilmaz and Gökmen (2013) and are within the range of results reported for six cultivars by Górnaś et al. (2016). Although the value obtained for total C18:3 (0.2%) in the current study is comparable to the one reported by Matthäus & Özcan, 2009 (0.1%), higher values of this fatty acid were published by some authors,

presenting ranges between 5.06 and 16.24% (Górnaś et al., 2016; Korlesky et al., 2016; Özcan, et. al., 2015; Yilmaz & Gökmen, 2013). Specifically in this study, results revealed the presence of one fatty acid that was not previously reported by other authors, erucic acid, a fatty acid commonly found in seeds and oils of the Brassicaceae family such as rapeseed and mustard seeds and is associated with adverse health effects in animals. Considering this, low erucic acid rapeseed varieties, such as canola, have been developed over the years (EFSA, 2016). Vaidya and Choe (2011) and Rezkas, Wroniak and Rusinek (2015) only reported minor changes in erucic acid content after roasting of mustard seed oil and high oleic suggesting that the amount of erucic acid in RO is intrinsic to the pits and not due to the roasting process. As it can be observed from Górnaś et al. (2016) fatty acids distribution can change according to the different cherry cultivars. In this study a blend of Montmorency and Balaton pits were used so results could not be strictly compared to the Montmorency varieties, since the Balaton cultivar may be contributing to these differences in the fatty acid distribution. The amount of total sterols (5913 ppm) was greater than the values reported by Korlesky et al. (2016) and Górnaś et al. (2016). However, the distribution of these compounds was similar among the studies, being β- Sitosterol the predominant sterol. Δ5-Avenasterol and Campesterol were also present in significant concentrations. In addition we found significant amounts of Sitostanol.

The distribution of tocopherols in our samples corresponds well with the distributions presented by other authors with γ-tocopherol contributing to the highest proportion. The concentration of total tocopherols obtained was 1100 ppm which is higher than the range of results published by other authors between 240.2-525.2 ppm (Korlesky et. al, 2016;

Matthäus & Özcan, 2009; Yilmaz & Gökmen, 2013). Specifically the concentration of α-tocopherol in our study (60 ppm) was comparable to results published by Korlesky et al. (2016) and Matthäus and Özcan (2009) who reported averages between 61.0-94.9 ppm for this compound. Nevertheless, we obtained higher values for δ- and γ-tocopherol (233 and 807 ppm respectively) compared to results published by those authors which ranged between 38.9-88.9 ppm for δ- and 183.8-400.0 ppm for γ-tocopherol. Matthäus and Özcan (2009), also reported values for tocopherols in cherry pit oil, but their results for α- and δ-tocopherol were lower than the values obtained in the present study and in the previously cited ones (4.7 and 15.1 ppm respectively).

Table 6: Concentrations and percentages of fatty acids, sterols & stanols, and tocopherols in roasted cherry pit oil. The fatty acids distribution was analyzed in fresh roasted pit oil sample (RO) and after 12 weeks of storage (RO12).

Fatty Acids Distribution			**Sterols and Stanols**	**% of Total**	**Tocopherols**	**ppm**
Fatty Acids	**% in RO**	**% in RO12**				
C14:0	<0.1	<0.1	Cholesterol	0.1	α-	60
C16:0	6.6	6.6	24-Methylene-Cholesterol	<0.1	δ-	233
C16:1	0.4	0.4	Brassicasterol	<0.1	γ-	807
C17:0	0.1	0.1	Campesterol	3.3	**Total tocopherols (ppm)**	**1100**
C17:1	0.1	0.1	Campestanol	0.2		
C18:0	2.5	2.5	Stigmasterol	<0.1		
C18:1	43.1	43	Δ7- Campesterol	<0.1		
C18:2	36	35.8	Δ5,23 Stigmasterol	<0.1		
C18:3	0.2	0.2	Clerosterol	0.7		
C20:0	1.2	1.2	β-Sitosterol	78.8		
C20:1	0.5	0.5	Sitostanol	4.5		
C22:0	0.3	0.3	Δ5-Avenasterol	6.8		
C22:1	8.8	8.8	Δ5,24 Stigmasterol	1.3		
C24:0	0.2	0.2	Δ7-Stigmasterol	2		
Other	<0.1	0.1	Δ7-Avenasterol	1.8		
			Total sterols (ppm)	**5913**		

5. Conclusions

The expeller press demonstrated to be an effective method for extraction of cherry pit oil directly from stones. The moisture content and the heat treatment applied to the pits have a significant impact on yield and in sensory properties, but under the conditions evaluated these variables had minimal effect on the quality parameters. No amygdalin was detected in any of the oils obtained. After 12 weeks of storage, quality and sensory parameters remained relatively stable in the cherry pit oil. The fatty acids, tocopherols and sterols profiles, if erucic acid is removed, demonstrate that the cherry pit oil is rich in bioactive compounds and it could potentially have applications in the food and cosmetic industries.

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CHAPTER 3:

^{1}H-NMR FOR MELATONIN QUANTIFICATION IN AQUEOUS SOLUTIONS, SUPPLEMENTS AND CHERRY PRODUCTS

1. Abstract

Melatonin is a hormone associated with the regulation of the circadian rhythm and sleep cycles. The consumption of exogenous melatonin either by supplements or different fruits or plants is associated with the improvement of sleeping indicators. However, methods for melatonin determination are complex and time consuming. The objective of this study was to develop a fast method to quantify melatonin in different matrices utilizing ^{1}H-NMR. A set of standards in water (0.001 ppm to 100 ppm) and liquid and solid supplements were analyzed for melatonin. Different cherry cultivars and their corresponding juices were tested for this compound. Melatonin was extracted using ethyl acetate and the extracts were analyzed by ^{1}H-NMR. Concentrations between 1 and 100 ppm were accurately quantified in standards. Recovery rates of 1.0±0.1 were obtained for the standards and 1.3 ± 0.2 for the supplements. No melatonin was detected in the cherries or cherry juice.

2. Introduction

Melatonin, N-acetyl-5-methoxytryptamine, is a hormone secreted by the pineal gland of vertebrates (Reiter & Tan, 2002). This compound is also synthetized by bacteria, protozoa, plants, fungi and invertebrates (Hardeland, Pandi-Perumal, & Cardinali, 2006). Melatonin has been associated with the regulation of the circadian rhythm, sleep cycles, antioxidant activity, and free radical scavenging activity (Reiter & Tan, 2002). Different studies have reported sleep promoting effects of exogenous melatonin (Brzezinski et al., 2005). Attenburrow and Sharpley (1996) reported that one dose of 1 mg of melatonin, in middle aged subjects, improved parameters such as sleep time and sleep efficiency. Hughes and Badia (1997) reported that exogenous melatonin may be effective in promoting sleep during daytime. Most of these studies were conducted by supplementing exogenous melatonin such as crystalline melatonin or capsules.

Tart cherries (*Prunus cerasus*) and their products consumption has been associated with health-promoting benefits due to the presence of antioxidant and anti-inflammatory compounds. For instance, the consumption of tart cherry juice before exercising generated smaller increase in pain after a race in runners (Kuehl, Perrier, Elliot & Chesnutt, 2010). Another study reported that consumption of a cherry juice blend days before and after eccentric exercise reduced the symptoms of muscle damage (Connolly,, McHugh, Padilla-Zakour, Carlson, & Sayers, 2006). But more interestingly, cherry consumption has been reported to be effective in improving sleeping. Pigeon, Carr, Gorman and Perlis (2010) reported that the consumption of tart cherry juice had a modest beneficial effect in adults with insomnia. In addition a study on tart cherry juice concentrate reported that its consumption increases the melatonin circulation and that

modestly improved sleeping time and quality in adults without sleeping disorders (Howatson, Bell, Tallent, Middleton, McHugh, & Ellis, 2012). Considering the studies in exogenous melatonin, the improvement of sleeping due to the consumption of cherry juice may be not only associated with the presence of anti-inflammatory components but also with the presence of melatonin. Levels of melatonin found in Montmorency juice concentrate were approximately 1.42 μg ml^{-1} (Howatson et al., 2012). However, the amount found in tart cherries (*Prunus cerasus*) ranged between 0.001 and 0.020 μg g^{-1} (Reiter & Tan, 2002; Burkhardt, Tan, Manchester, Hardeland, & Reiter, 2001).

Different methods for extraction and quantification have been developed over the years to analyze melatonin in different matrices. Enzyme Linked Immunosorbent Assay (ELISA) and High Performance Liquid Chromatography equipped with Fluorescence or Mass Spectroscopy detectors (HPLC-FLD or HPLC-MS) are some of the techniques applied by different authors (Burkhardt, et al., 2001; Cao, Murch O'Brien, & Saxena, 2006; Pape, & Lüning, 2006). Gas Chromatography with Mass detection (GC-MS) can be utilized for melatonin quantification (Kennaway, Frith, Phillipou, Matthews, & Seamark, 1977). However, these methods have limitations specifically related to the sample preparation as some of these procedures require sample destruction or compound derivatization, which may add complexity to the analyses and affect the recovery rate of the compound. Moreover, chromatography and immunoassays methods were reported not to be reliable on their own, since interferences may create false positives or give erroneous results (Van Tassel & O'Neill, 2001). Finding a fast and reliable technique to quantify melatonin in different matrices is of vital importance to get accurate results.

The objective of this study was to develop a method to quantify melatonin in different matrices utilizing ^{1}H-NMR. The method was verified by testing it in melatonin supplements. The method was also evaluated for direct quantification of melatonin in two cherry cultivars and their corresponding juices, as well as its effectiveness in a complex food matrix.

3. Materials and methods

3.1. Reagents

Deuterium Oxide (D_2O, D 99.9%) and 0.02% (W/V) Trimethylsilylpropanoic acid (TMSP, 2,2,3,3-D_4 98%) in D_2O (99.9%), TMSP, were obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Melatonin (>98%) was obtained from Sigma Aldrich (St. Louis, MO, USA).

3.2. Samples

Three different commercial melatonin supplements, in liquid and tablet form, were purchased in grocery stores and pharmacies in Ithaca, NY: 1 mg in 4 ml, 5 mg in 15 ml, and 3 mg tablets.

Frozen cherries and juice samples were also analyzed. Two different cultivars of frozen tart cherries (*Prunus cerasus*) were obtained from a cherry juice manufacturing facility located in Northeastern New York. The two cultivars were Montmorency and Balaton. Tart cherry juice samples were also obtained from this facility. The juices were produced by commercially processing a blend of these two cherry cultivars, including

heating the fruit up to 79ºC. Once that temperature was reached, the pits were removed by sending the juice to a finisher. Juices were packed in capped PET bottles.

3.3. Preparation of melatonin standards

Different solutions of melatonin in deionized water were prepared. An initial 100 µg/g (100 ppm) melatonin stock solution was made. Afterwards successive dilutions of 10, 1, 0.1, 0.01 and 0.001 melatonin in cherry juice were made. Ten grams of each solution were placed in 50 ml centrifuge tubes and extracted as described in 3.6. Experiments were conducted in triplicate.

3.4. Sample preparation for supplements

A specific volume of the two liquid supplements was added directly into 50 ml centrifuge tubes. The volume added was estimated to be equivalent to 1 mg of melatonin. For solid tablets, 1 tablet was placed in a centrifuge and taking it to a total weight of 30 g with deionized water. The tablet with the water were mixed together until all the tablet was dissolved. Ten grams of this solution were placed in a 50 ml centrifuge tube. All the samples were then extracted as described in 3.6 and analyzed by ^{1}H-NMR as described in 3.7. All the studies were conducted in triplicate.

3.5. Cherry products sample preparation

The frozen cherries were thawed under refrigeration, pitted and juiced in a Braun multipress automatic kitchen juicer (Aschaffenburg, Germany). Juices were then filtered using 0.215 mm coarse filter papers. Ten grams of each of these different fresh

cherry juices and from the processed juice described in 3.2. were weighed into 50 ml centrifuge tubes and extracted as described in 3.4. and analyzed by ^{1}H-NMR as described in 3.5. All the samples were analyzed in triplicate.

3.6. Melatonin extraction

Five ml of ethyl acetate were added to the centrifuge tubes with the corresponding volume of standard (3.3) or sample (3.4 and 3.5). Samples were mixed in a vortex for 1 min and afterwards they were centrifuged for 5 min at 5000 rpm. The ethyl acetate supernantant was transferred to another centrifuge tube. The same procedure was repeated three more times combining all the supernatants together. Samples were dried in a Nitrogen blowing station equipped with a water bath set up at 37ºC and with a nitrogen pressure of 15 psi (103.4 kPa). Extracts were then diluted with 300 or 350 µl of D_2O and 100 µl of TMSP. Diluted extracts were mixed in a vortex mixer for 10 s and sonicated for other 20 s. Samples were transferred to the 5 mm Precision NMR tubes and analyzed as described in 3.7.

3.7. Melatonin determination by ^{1}H- NMR

Samples were analyzed on a Varian I-NOVA NMR instrument operating at a frequency of 600 MHz. A one-dimensional ^{1}H-NMR spectra was recorded at 25ºC with 1.708 s acquisition time, a pulse angle of 90º and a relaxation delay of 30 s. Four scans were run in samples with concentrations equal or above 1 µg/g and between 32 and 512 scans were run for samples with lower concentrations. Results were analyzed with MestReNova (Version 11.0) by manually phasing, baseline correcting and integrating

the spectra. Chemical shifts were standardized by adjusting the resonance signal from the silyl methyl protons of TMSP at 0.00 ppm.

4. Results and discussion

4.1. Melatonin detection and quantification in water solutions

Melatonin was quantifiable in water solutions by ^{1}H- NMR in concentrations between 1 and 100 ppm. At 0.1 ppm, melatonin was detectable but not quantifiable. Considering this, concentrations below 0.1 ppm were not analyzed in the ^{1}H-NMR. The hydrogens selected for quantification in the ^{1}H-NMR spectra are the ones corresponding to the first methyl group in the melatonin which gave a sharp, clear peak to identify and quantify in a non-crowded area of the spectra (Figure 1). This peak is present around a frequency of 1.89 ppm. Table 1 shows the recovery rates obtained for each standard analyzed. In contrast to HPLC-FLD or ELISA, in which recoveries can range from 60% to 83% (Burkhardt, et al., 2001; Pape, & Lüning, 2006; Roopin, Jacobi, & Levy, 2013), in this study melatonin recovery was around 100%.

Table 1: Recovery rates for melatonin standards. Results are expressed as mean ± SD (n=3).

Melatonin ppm (μg g^{-1})	Recovery Rates
100	1.1 ± 0.0
10	1.0 ± 0.1
1	1.0 ± 0.2
0.1	Detectable

This recovery rate also suggests that ethyl acetate is effective for melatonin extraction in concentrations of 100 ppm or lower. Furthermore, in contrast to GC-MS or HPLC-MS, in which sample derivatization is required, samples analyzed by ^{1}H-NMR have minimum sample preparation and melatonin is directly observed in the spectra.

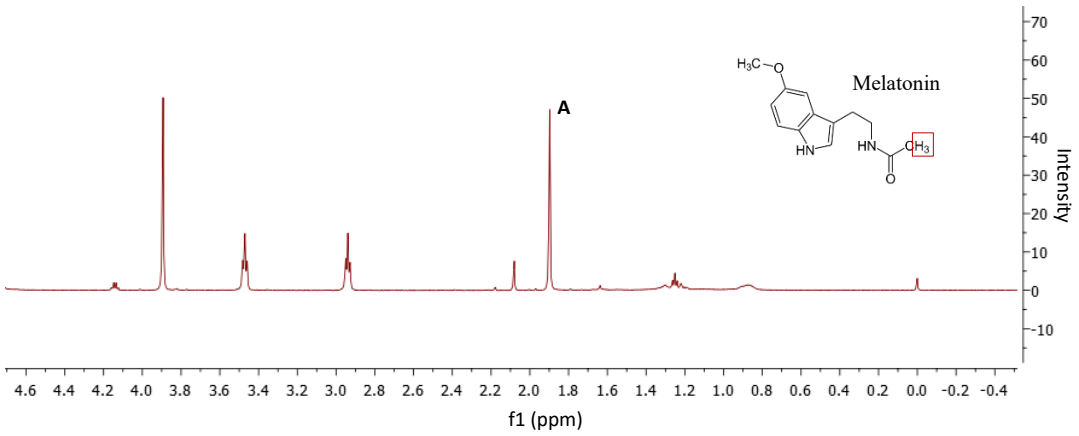

Figure 1: ^{1}H-NMR Spectra for the 100 ppm melatonin standard. (A) Corresponds to the hydrogens selected for quantification (hydrogens in the methyl group of the melatonin molecule).

4.2. Melatonin quantification in commercial supplements

To verify the effectiveness of the method developed in different matrices and to assess the melatonin concentration declared in commercial products, this compound was quantified in commercial supplements. Table 2 displays the results obtained for the different supplements analyzed. The melatonin peak was clear and quantifiable, either in the liquid or solid matrices analyzed, appearing at 1.89 ppm as expected. As a result, it can be confirmed that the ^{1}H-NMR method is effective for melatonin quantification in liquid and solid products formulated with few ingredients or components. Melatonin values obtained in all the supplements where higher than the amount of melatonin declared in the package (on average 40% higher for liquid supplements and 10% higher

for the tablet). Considering this, it can be concluded that the results obtained confirmed that the supplements analyzed were in compliance with the label declaration.

Table 2: ^{1}H-NMR results for melatonin supplements. Results are expressed as mean ± SD (n=3).

Matrix	Melatonin declared in label	Volume sampled (ml)	Expected grams	^{1}H-NMR Result (g)
Liquid	5 mg in 15 ml	3	0.0010	0.0014 ± 0.0001
Liquid	1 mg in 4 ml	4	0.0010	0.0014 ± 0.0000
Tablets	3 mg in 1 tablet (1 tablet diluted in 30 g of water)	10	0.0010	0.0011 ± 0.0001

4.3 Melatonin in cherry products

With the objective of testing the method in a more complex matrix and in lower concentrations, two different cherry cultivars and their corresponding commercially processed juice were analyzed. No melatonin was detected in Balaton or Montmorency cherries or in their commercially processed juice. Figure 2 shows the spectras of the Montmorency cherry, the 100 ppm standard and the liquid melatonin supplement. From the figure it can be observed that while melatonin is clearly identified in the standard and supplement, it is not detectable in the cherry sample. Burkhardt et al. (2001) reported the presence of melatonin in cherries in concentrations that ranged between 1.07-2.18 ng g^{-1} (0.001 – 0.002 µg g^{-1}) for Balaton cherries and between 5.57-19.59 ng g^{-1} (0.005 – 0.02 µg g^{-1}) for Montmorency cherries. As previously described, the method developed in this study is able to detect up to 0.1 µg g^{-1} and quantify from 1 µg g^{-1} onwards. Considering this limitation, it cannot be concluded that melatonin is not present in the cherry products, since the method designed can only quantify concentrations of at least 1 ppm, which is a much higher value than results previously reported for these products. These results confirm that although the ^{1}H-NMR is a fast

and reliable method for melatonin quantification that requires little sample preparation, it cannot be applied for the quantification of melatonin below 1 ppm. Nevertheless, the spectra looks clear in the area corresponding to the melatonin peak suggesting that if cherries and juices samples are concentrated enough, melatonin may be detectable in this stone fruit by ^{1}H-NMR.

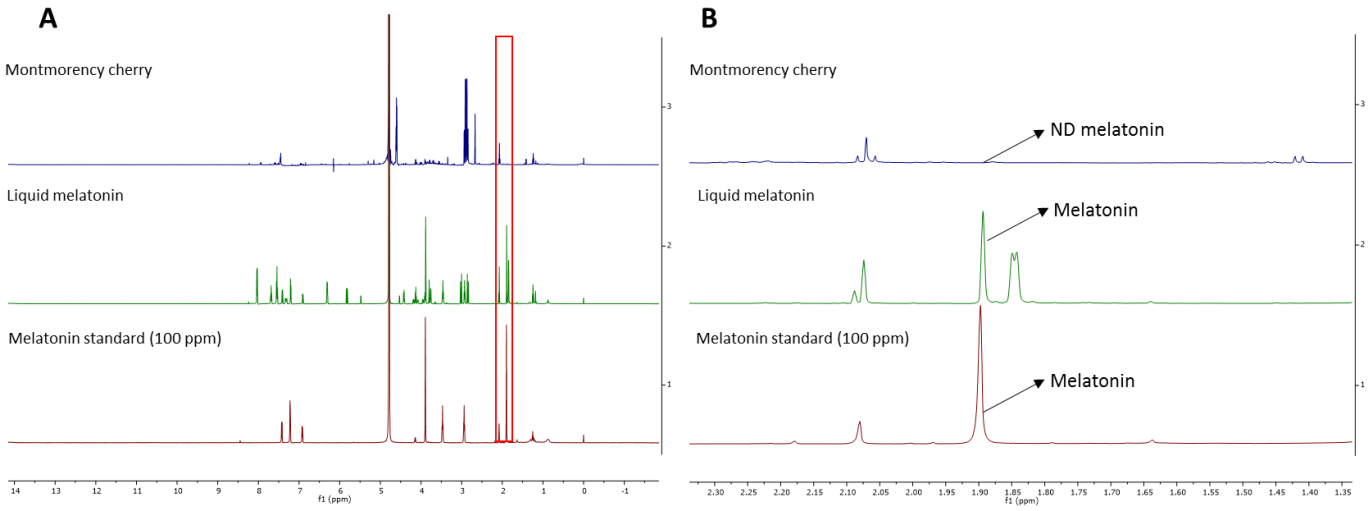

Figure 2: (A) ^{1}H-NMR Spectra for Montmorency cherry, liquid melatonin supplement and 100 ppm melatonin standard. Melatonin expected area is marked. (B) Amplified Spectra for the melatonin area. Melatonin is clearly identified in the standard and liquid supplement but is not detected in the Montmorency cherry.

5. Conclusions

^{1}H-NMR proved to be a useful method for the direct detection and quantification of melatonin in different matrices in concentrations higher than 0.1 ppm. Melatonin supplements were analyzed for their melatonin content and all of them were in compliance with the concentration declared in the label. No melatonin was detected in cherries or cherry juice. This may be associated with the detection limit of the method applied.

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APPENDIX 1: CYANOGENIC GLYCOSIDES RESULTS AND CHERRIES WEIGHTS

Table 1: Cyanogenic Glycosides content in cherry kernels and shells obtained by HPLC and ^{1}H-NMR.

Trials	Variety	Part	CG	HPLC (µg amygdalin/g dry matter)	NMR (µg amygdalin/g dry matter)	HPLC (µg amygdalin/g fresh matter)	NMR (µg amygdalin/g fresh matter
1st	Balaton	Kernel	Amygdalin	22274	22237	12901	12880
1st	Balaton	Kernel	Amygdalin	22461	21071	13010	12204
1st	Balaton	Kernel	Amygdalin	21533	20104	12472	11645
1st	Balaton	Shell	Amygdalin	ND	ND	ND	ND
1st	Balaton	Shell	Amygdalin	ND	ND	ND	ND
1st	Balaton	Shell	Amygdalin	ND	ND	ND	ND
1st	Balaton	Kernel	Prunasin	ND	ND	ND	ND
1st	Balaton	Kernel	Prunasin	ND	ND	ND	ND
1st	Balaton	Kernel	Prunasin	ND	ND	ND	ND
2nd	Balaton	Kernel	Amygdalin	24705	24453	17034	16860
2nd	Balaton	Kernel	Amygdalin	24152	23725	16653	16358
2nd	Balaton	Kernel	Amygdalin	24386	24406	16814	16828
2nd	Balaton	Shell	Amygdalin	ND	ND	ND	ND
2nd	Balaton	Shell	Amygdalin	ND	ND	ND	ND
2nd	Balaton	Shell	Amygdalin	ND	ND	ND	ND
2nd	Balaton	Kernel	Prunasin	ND	ND	ND	ND
2nd	Balaton	Kernel	Prunasin	ND	ND	ND	ND
2nd	Balaton	Kernel	Prunasin	ND	ND	ND	ND
3rd	Balaton	Kernel	Amygdalin	22755	24151	15238	16173
3rd	Balaton	Kernel	Amygdalin	22993	24476	15398	16390
3rd	Balaton	Kernel	Amygdalin	21441	21051	14358	14097
3rd	Balaton	Shell	Amygdalin	ND	ND	ND	ND
3rd	Balaton	Shell	Amygdalin	ND	ND	ND	ND
3rd	Balaton	Shell	Amygdalin	ND	ND	ND	ND
3rd	Balaton	Kernel	Prunasin	ND	ND	ND	ND
3rd	Balaton	Kernel	Prunasin	ND	ND	ND	ND
3rd	Balaton	Kernel	Prunasin	ND	ND	ND	ND
1st	Montmorency	Kernel	Amygdalin	10328	8970	5775	5016
1st	Montmorency	Kernel	Amygdalin	10056	9249	5623	5172
1st	Montmorency	Kernel	Amygdalin	9854	9638	5510	5389
1st	Montmorency	Shell	Amygdalin	ND	ND	ND	ND
1st	Montmorency	Shell	Amygdalin	ND	ND	ND	ND
1st	Montmorency	Shell	Amygdalin	ND	ND	ND	ND
1st	Montmorency	Kernel	Prunasin	ND	ND	ND	ND

1st	Montmorency	Kernel	Prunasin	ND	ND	ND	ND
1st	Montmorency	Kernel	Prunasin	ND	ND	ND	ND
2nd	Montmorency	Kernel	Amygdalin	12485	12733	7424	7571
2nd	Montmorency	Kernel	Amygdalin	12747	12269	7579	7295
2nd	Montmorency	Kernel	Amygdalin	12952	11933	7701	7096
2nd	Montmorency	Shell	Amygdalin	ND	ND	ND	ND
2nd	Montmorency	Shell	Amygdalin	ND	ND	ND	ND
2nd	Montmorency	Shell	Amygdalin	ND	ND	ND	ND
2nd	Montmorency	Kernel	Prunasin	ND	ND	ND	ND
2nd	Montmorency	Kernel	Prunasin	ND	ND	ND	ND
2nd	Montmorency	Kernel	Prunasin	ND	ND	ND	ND
3rd	Montmorency	Kernel	Amygdalin	16815	18766	11028	12308
3rd	Montmorency	Kernel	Amygdalin	18402	18316	12069	12013
3rd	Montmorency	Kernel	Amygdalin	18559	18326	12172	12019
3rd	Montmorency	Shell	Amygdalin	ND	ND	ND	ND
3rd	Montmorency	Shell	Amygdalin	ND	ND	ND	ND
3rd	Montmorency	Shell	Amygdalin	ND	ND	ND	ND
3rd	Montmorency	Kernel	Prunasin	ND	ND	ND	ND
3rd	Montmorency	Kernel	Prunasin	ND	ND	ND	ND
3rd	Montmorency	Kernel	Prunasin	ND	ND	ND	ND
1st	Processed	Kernel	Amygdalin	4193	3747	2470	2207
1st	Processed	Kernel	Amygdalin	4015	3919	2365	2309
1st	Processed	Kernel	Amygdalin	3823	3213	2252	1893
1st	Processed	Shell	Amygdalin	ND	ND	ND	ND
1st	Processed	Shell	Amygdalin	ND	ND	ND	ND
1st	Processed	Shell	Amygdalin	ND	ND	ND	ND
1st	Processed	Kernel	Prunasin	ND	ND	ND	ND
1st	Processed	Kernel	Prunasin	ND	ND	ND	ND
1st	Processed	Kernel	Prunasin	ND	ND	ND	ND
2nd	Processed	Kernel	Amygdalin	1828	1789	1077	1054
2nd	Processed	Kernel	Amygdalin	2367	1656	1394	976
2nd	Processed	Kernel	Amygdalin	2443	2048	1439	1207
2nd	Processed	Shell	Amygdalin	ND	ND	ND	ND
2nd	Processed	Shell	Amygdalin	ND	ND	ND	ND
2nd	Processed	Shell	Amygdalin	ND	ND	ND	ND
2nd	Processed	Kernel	Prunasin	ND	ND	ND	ND
2nd	Processed	Kernel	Prunasin	ND	ND	ND	ND
2nd	Processed	Kernel	Prunasin	ND	ND	ND	ND
3rd	Processed	Kernel	Amygdalin	3302	2543	1951	1503
3rd	Processed	Kernel	Amygdalin	3605	3411	2130	2016
3rd	Processed	Kernel	Amygdalin	4357	3559	2575	2103
3rd	Processed	Shell	Amygdalin	ND	ND	ND	ND

3rd	Processed	Shell	Amygdalin	ND	ND	ND	ND
3rd	Processed	Shell	Amygdalin	ND	ND	ND	ND
3rd	Processed	Kernel	Prunasin	ND	ND	ND	ND
3rd	Processed	Kernel	Prunasin	ND	ND	ND	ND
3rd	Processed	Kernel	Prunasin	ND	ND	ND	ND

Table 2: HCN Equivalents in cherry kernels and shells obtained by HPLC and ^{1}H-NMR.

Trials	Variety	Part	CG	HPLC HCN (µg/g dry kernell)	NMR HCN (µg/g dry kernell)	HPLC HCN (µg/g fresh kernell)	NMR HCN (µg/freshkernell)	HCN in 1 kernel
1st	Balaton	Kernel	Amygdalin	1316	1314	762	761	68.8
1st	Balaton	Kernel	Amygdalin	1327	1245	769	721	69.4
1st	Balaton	Kernel	Amygdalin	1272	1188	737	688	66.5
1st	Balaton	Shell	Amygdalin	ND	ND	ND	ND	ND
1st	Balaton	Shell	Amygdalin	ND	ND	ND	ND	ND
1st	Balaton	Shell	Amygdalin	ND	ND	ND	ND	ND
1st	Balaton	Kernel	Prunasin	ND	ND	ND	ND	ND
1st	Balaton	Kernel	Prunasin	ND	ND	ND	ND	ND
1st	Balaton	Kernel	Prunasin	ND	ND	ND	ND	ND
2nd	Balaton	Kernel	Amygdalin	1460	1445	1006	996	80.0
2nd	Balaton	Kernel	Amygdalin	1427	1402	984	966	78.2
2nd	Balaton	Kernel	Amygdalin	1441	1442	993	994	79.0
2nd	Balaton	Shell	Amygdalin	ND	ND	ND	ND	ND
2nd	Balaton	Shell	Amygdalin	ND	ND	ND	ND	ND
2nd	Balaton	Shell	Amygdalin	ND	ND	ND	ND	ND
2nd	Balaton	Kernel	Prunasin	ND	ND	ND	ND	ND
2nd	Balaton	Kernel	Prunasin	ND	ND	ND	ND	ND
2nd	Balaton	Kernel	Prunasin	ND	ND	ND	ND	ND
3rd	Balaton	Kernel	Amygdalin	1344	1427	900	956	67.3
3rd	Balaton	Kernel	Amygdalin	1358	1446	910	968	68.0
3rd	Balaton	Kernel	Amygdalin	1267	1244	848	833	63.4
3rd	Balaton	Shell	Amygdalin	ND	ND	ND	ND	ND
3rd	Balaton	Shell	Amygdalin	ND	ND	ND	ND	ND
3rd	Balaton	Shell	Amygdalin	ND	ND	ND	ND	ND
3rd	Balaton	Kernel	Prunasin	ND	ND	ND	ND	ND
3rd	Balaton	Kernel	Prunasin	ND	ND	ND	ND	ND
3rd	Balaton	Kernel	Prunasin	ND	ND	ND	ND	ND
1st	Montmorency	Kernel	Amygdalin	610	530	341	296	26.1
1st	Montmorency	Kernel	Amygdalin	594	546	332	306	25.4
1st	Montmorency	Kernel	Amygdalin	582	569	326	318	24.9
1st	Montmorency	Shell	Amygdalin	ND	ND	ND	ND	ND
1st	Montmorency	Shell	Amygdalin	ND	ND	ND	ND	ND

1st	Montmorency	Shell	Amygdalin	ND	ND	ND	ND	ND
1st	Montmorency	Kernel	Prunasin	ND	ND	ND	ND	ND
1st	Montmorency	Kernel	Prunasin	ND	ND	ND	ND	ND
1st	Montmorency	Kernel	Prunasin	ND	ND	ND	ND	ND
2nd	Montmorency	Kernel	Amygdalin	738	752	439	447	28.7
2nd	Montmorency	Kernel	Amygdalin	753	725	448	431	29.3
2nd	Montmorency	Kernel	Amygdalin	765	705	455	419	29.8
2nd	Montmorency	Shell	Amygdalin	ND	ND	ND	ND	ND
2nd	Montmorency	Shell	Amygdalin	ND	ND	ND	ND	ND
2nd	Montmorency	Shell	Amygdalin	ND	ND	ND	ND	ND
2nd	Montmorency	Kernel	Prunasin	ND	ND	ND	ND	ND
2nd	Montmorency	Kernel	Prunasin	ND	ND	ND	ND	ND
2nd	Montmorency	Kernel	Prunasin	ND	ND	ND	ND	ND
3rd	Montmorency	Kernel	Amygdalin	993	1109	652	727	32.3
3rd	Montmorency	Kernel	Amygdalin	1087	1082	713	710	35.4
3rd	Montmorency	Kernel	Amygdalin	1096	1083	719	710	35.7
3rd	Montmorency	Shell	Amygdalin	ND	ND	ND	ND	ND
3rd	Montmorency	Shell	Amygdalin	ND	ND	ND	ND	ND
3rd	Montmorency	Shell	Amygdalin	ND	ND	ND	ND	ND
3rd	Montmorency	Kernel	Prunasin	ND	ND	ND	ND	ND
3rd	Montmorency	Kernel	Prunasin	ND	ND	ND	ND	ND
3rd	Montmorency	Kernel	Prunasin	ND	ND	ND	ND	ND
1st	Processed	Kernel	Amygdalin	248	221	146	130	10.9
1st	Processed	Kernel	Amygdalin	237	232	140	136	10.5
1st	Processed	Kernel	Amygdalin	226	190	133	112	10.0
1st	Processed	Shell	Amygdalin	ND	ND	ND	ND	ND
1st	Processed	Shell	Amygdalin	ND	ND	ND	ND	ND
1st	Processed	Shell	Amygdalin	ND	ND	ND	ND	ND
1st	Processed	Kernel	Prunasin	ND	ND	ND	ND	ND
1st	Processed	Kernel	Prunasin	ND	ND	ND	ND	ND
1st	Processed	Kernel	Prunasin	ND	ND	ND	ND	ND
2nd	Processed	Kernel	Amygdalin	108	106	64	62	5.6
2nd	Processed	Kernel	Amygdalin	140	98	82	58	7.3
2nd	Processed	Kernel	Amygdalin	144	121	85	71	7.5
2nd	Processed	Shell	Amygdalin	ND	ND	ND	ND	ND
2nd	Processed	Shell	Amygdalin	ND	ND	ND	ND	ND
2nd	Processed	Shell	Amygdalin	ND	ND	ND	ND	ND
2nd	Processed	Kernel	Prunasin	ND	ND	ND	ND	ND
2nd	Processed	Kernel	Prunasin	ND	ND	ND	ND	ND
2nd	Processed	Kernel	Prunasin	ND	ND	ND	ND	ND
3rd	Processed	Kernel	Amygdalin	195	150	115	89	10.7
3rd	Processed	Kernel	Amygdalin	213	202	126	119	11.7

3rd	Processed	Kernel	Amygdalin	257	210	152	124	14.2
3rd	Processed	Shell	Amygdalin	ND	ND	ND	ND	ND
3rd	Processed	Shell	Amygdalin	ND	ND	ND	ND	ND
3rd	Processed	Shell	Amygdalin	ND	ND	ND	ND	ND
3rd	Processed	Kernel	Prunasin	ND	ND	ND	ND	ND
3rd	Processed	Kernel	Prunasin	ND	ND	ND	ND	ND
3rd	Processed	Kernel	Prunasin	ND	ND	ND	ND	ND

Table 3: Cyanogenic Glycosides Content and HCN equivalents in Cherry Flesh and Processed Juice obtained by ^{1}H-NMR.

Variety	CG	Trial	NMR- mg amygdalin/ g juice	NMR-mg HCN /kg juice
Montmorency	Amygdalin	1st	0.07	3.98
Montmorency	Amygdalin	2nd	0.06	3.25
Montmorency	Amygdalin	3rd	0.08	4.76
Balaton	Amygdalin	1st	0.09	5.33
Balaton	Amygdalin	2nd	0.09	5.21
Balaton	Amygdalin	3rd	0.1	5.7
Processed	Amygdalin	1st	0.06	3.78
Processed	Amygdalin	2nd	0.05	2.67
Processed	Amygdalin	3rd	0.07	4.11

Table 4: Cherries Weights.

Trial 1						Trial 2						Trial 3					
Balaton		Montmorency		Processed		Balaton		Montmorency		Processed		Balaton		Montmorency		Processed	
Kernell	Shell	Kernell	Shell	Kernell	Shell	Kernell	Shell	Kernell	Shell	Kernell	Shell	Kernell	Shell	Kernell	Shell	Kernell	Shell
0.0932	0.1461	0.1252	0.1806	0.0976	0.1379	0.0795	0.252	0.0854	0.1873	0.1011	0.1551	0.0033	0.2117	0.0588	0.1355	0.1038	0.178
0.0787	0.1219	0.0923	0.1447	0.0896	0.1802	0.094	0.1714	0.073	0.1516	0.0962	0.2743	0.0981	0.25344	0.0542	0.1575	0.084	0.1955
0.1228	0.2655	0.1111	0.1365	0.0568	0.1303	0.0657	0.1871	0.0986	0.2321	0.0254	0.212	0.1086	0.199	0.073	0.2163	0.0986	0.131
0.1176	0.1859	0.0556	0.1348	0.1057	0.1599	0.0635	0.1961	0.0937	0.2078	0.0668	0.1732	0.0946	0.1967	0.0612	0.1568	0.127	0.2178
0.1212	0.1919	0.0486	0.2146	0.0254	0.1792	0.1156	0.2067	0.0763	0.1655	0.1144	0.1966	0.091	0.2378	0.0383	0.2121	0.1121	0.2169
0.0463	0.2193	0.1143	0.189	0.035	0.2299	0.0957	0.1616	0.0251	0.2387	0.1004	0.1695	0.08886	0.228	0.0559	0.1046	0.0346	0.2282
0.01155	0.1786	0.1176	0.159	0.0892	0.1695	0.0841	0.1897	0.0548	0.1998	0.0833	0.206	0.0121	0.1922	0.0545	0.1979	0.1084	0.1981
0.0945	0.1864	0.0292	0.1813	0.0106	0.2049	0.0747	0.1539	0.0857	0.2099	0.1547	0.2618	0.0037	0.1578	0.0659	0.1717	0.0753	0.1944
0.0932	0.1405	0.1242	0.1656	0.0979	0.2004	0.1015	0.2236	0.1024	0.1877	0.1125	0.2034	0.113	0.2382	0.0701	0.1232	0.0712	0.1471
0.1044	0.1957	0.0295	0.1437	0.1257	0.1853	0.1211	0.2177	0.0746	0.2035	0.0952	0.1615	0.0938	0.2252	0.0626	0.1517	0.0826	0.1515
0.0998	0.2317	0.0946	0.1317	0.0883	0.1852	0.0776	0.1966	0.066	0.1567	0.1245	0.276	0.0398	0.2171	0.0607	0.1218	0.0796	0.1425
0.1186	0.1659	0.0527	0.1436	0.068	0.1162	0.1102	0.159	0.0941	0.1887	0.0805	0.1226	0.1276	0.2308	0.0646	0.1541	0.0358	0.2084
0.0974	0.1524	0.0194	0.1306	0.1205	0.2111	0.0832	0.1672	0.0107	0.1467	0.0623	0.151	0.0815	0.1545	0.017	0.1843	0.1208	0.202
0.0727	0.159	0.0828	0.1264	0.053	0.1683	0.1092	0.1957	0.0534	0.164	0.0949	0.1912	0.1022	0.2134	0.0761	0.1269	0.0279	0.2269
0.1008	0.1737	0.1075	0.1473	0.0149	0.151	0.0941	0.2486	0.0535	0.1297	0.1714	0.2805	0.1187	0.2499	0.0623	0.1288	0.0305	0.1908
0.0717	0.205	0.0958	0.1327	0.0722	0.2098	0.01	0.239	0.0522	0.221	0.0975	0.1962	0.0987	0.193	0.0844	0.145	0.0831	0.1891
0.1009	0.1788	0.0108	0.1544	0.0422	0.1985	0.1032	0.2082	0.0852	0.1439	0.0758	0.1261	0.0876	0.2553	0.0607	0.1553	0.0937	0.2014
0.1113	0.1726	0.1188	0.2015	0.0544	0.1387	0.055	0.1691	0.0901	0.1876	0.0249	0.2003	0.047	0.254	0.0042	0.1501	0.115	0.2049
0.0991	0.222	0.0665	0.1441	0.0093	0.1298	0.0705	0.136	0.0312	0.1573	0.1427	0.3035	0.0939	0.2122	0.0796	0.1625	0.1174	0.2131
0.0991	0.1463	0.0641	0.1492	0.0918	0.1936	0.0984	0.2002	0.0852	0.2032	0.1101	0.1996	0.0854	0.1544	0.035	0.2031	0.1223	0.2236
0.0674	0.1352	0.1062	0.1429	0.0955	0.1651	0.0589	0.1185	0.0136	0.1789	0.0714	0.1524	0.0592	0.2825	0.0659	0.1644	0.1051	0.2207
0.0905	0.1636	0.0807	0.1485	0.1025	0.2153	0.067	0.1431	0.0591	0.2731	0.0581	0.2255	0.0404	0.1753	0.0154	0.1742	0.117	0.1888
0.0924	0.1482	0.0955	0.1335	0.0368	0.1901	0.0578	0.0984	0.0275	0.1153	0.0696	0.1637	0.0829	0.1669	0.0093	0.1326	0.1042	0.2133
0.0949	0.1769	0.037	0.1293	0.124	0.2095	0.0834	0.1575	0.0835	0.1752	0.0729	0.1421	0.0772	0.1885	0.0671	0.1751	0.0942	0.1643
0.098	0.1844	0.1033	0.1752	0.0831	0.133	0.0878	0.1614	0.012	0.1763	0.1011	0.2155	0.0785	0.1997	0.0241	0.18	0.0872	0.2173
0.0998	0.1692	0.1077	0.1659	0.104	0.2395	0.008	0.1558	0.1276	0.155	0.1052	0.2163	0.0327	0.2564	0.0594	0.1389	0.0761	0.1698
0.0817	0.1419	0.0186	0.125	0.0686	0.2113	0.0809	0.1553	0.0202	0.1811	0.0788	0.1162	0.1182	0.2364	0.0078	0.1727	0.0942	0.1991
0.0507	0.2109	0.0543	0.1406	0.091	0.1717	0.0102	0.16	0.0961	0.1575	0.0835	0.1701	0.0629	0.1793	0.0471	0.1161	0.0901	0.1825
0.09	0.2197	0.0459	0.1487	0.0824	0.1337	0.132	0.2284			0.057	0.1168	0.0942	0.1684	0.0296	0.1336	0.0575	0.1996
0.0906	0.1873	0.0955	0.129	0.097	0.1252	0.0917	0.1837			0.1328	0.1979	0.0057	0.149	0.0662	0.1291	0.1105	0.2136
0.0894	0.1561	0.0672	0.1785	0.0845	0.153					0.1252	0.2193			0.0076	0.2019		

Table 5: Moisture loss in different cherry components during freeze-drying.

	Balaton		Montmorency		Processed	
	Kernel	Shell	Kernel	Shell	Kernel	Shell
Trial 1	39%	25%	42%	25%	38%	24%
Trial 2	27%	20%	31%	27%	34%	28%
Trial 3	30%	24%	40%	29%	38%	25%

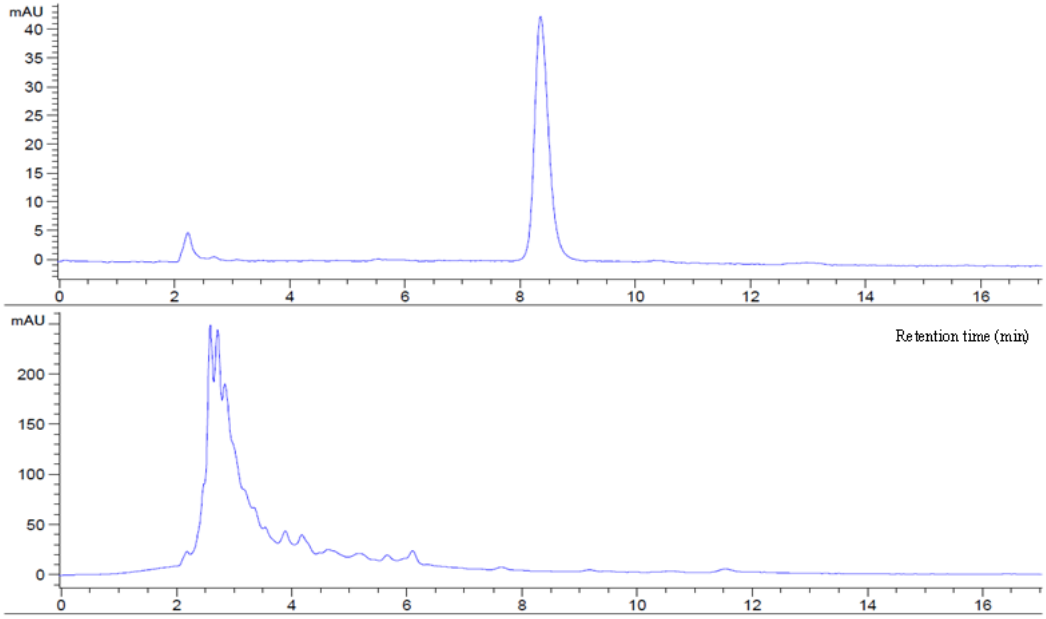

Figure 1: HPLC Chromatogram for cherry shells (A) Amygdalin standard. (B) Balaton shell- no amygdalin detected.

APPENDIX 2: ANALYSIS OF CHERRY PIT OIL

Table 1: Quality variables and amygdalin concentration results in different cherry pit oils.

Oil	L*	a*	b*	Moisture and Volatiles (%)	Peroxide Value (mEq Peroxides/kg)	Free Fatty Acids (% Oleic Acid)	Refraction Index	Amygdalin cc (µg amygdalin/ g)	Viscosity 25°C (cP)
D1O (1)	67.73	12.37	74.54	0.47	1.40	0.53	1.47678	ND	128
D1O (2)	67.74	12.35	74.58	0.46	1.34	0.53	1.47676	ND	128
D1O (3)	67.77	12.37	74.79	0.50	1.34	0.54	1.47674	ND	127
D2O (1)	67.1	13.09	74.04	0.46	1.19	0.50	1.47764	ND	125
D2O (2)	67.11	13.07	73.94	0.45	1.19	0.47	1.47767	ND	125
D2O (3)	67.13	13.04	73.7	0.46	1.14	0.51	1.47763	ND	125
RO (1)	65.45	14.03	71.15	0.37	0.99	0.47	1.47725	ND	128
RO (2)	65.51	14.01	71.14	0.35	0.98	0.44	1.47724	ND	128
RO (3)	65.53	13.99	71.18	0.38	1.01	0.47	1.47725	ND	127

Table 2: Shelf life study results for roasted cherry pit oils.

Week	L*	a*	b*	Moisture and Volatiles (%)	Peroxides Value (mEq peroxide/ kg)	Free fatty acids (% Oleic Acid)	Refractive Index
0 (1)	65.45	14.03	71.15	0.37	0.99	0.47	1.47725
0 (2)	65.51	14.01	71.14	0.36	0.98	0.44	1.47724
0 (3)	65.53	13.99	71.18	0.40	1.01	0.47	1.47725
4 (1)	65.29	13.75	70.82	0.37	1.03	0.48	1.47785
4 (2)	65.21	13.78	70.73	0.35	0.93	0.46	1.47779
4 (3)	65.22	13.78	70.99	0.38	1.01	0.45	1.47783
8 (1)	64.81	14.46	69.74	0.36	1.12	0.43	1.47784
8 (2)	64.74	14.46	69.57	0.39	1.10	0.42	1.47797
8 (3)	64.91	14.24	69.84	0.37	1.01	0.44	1.47792
12 (1)	64.79	14.53	69.87	0.34	1.29	0.43	1.4776
12 (2)	64.77	14.6	70.11	0.32	1.34	0.42	1.47772
12 (3)	64.78	14.4	70.03	0.33	1.33	0.41	1.47773

Table 3: Moisture content in cherry pits.

Fresh	D1	D2	R
35.36	9.01	7.84	10.91
35.84	8.88	8.13	9.98
35.21	8.72	7.38	9.92
33.86	8.48	8.42	9.9
35.58	8.45	7.42	9.87
34.23	8.2	8.14	10.09
36.04	8.67	8.06	10.64
35.57	8.53	8.17	10.32
35.99	8.9	7.46	10.06
34.83	8.56	7.42	10.7

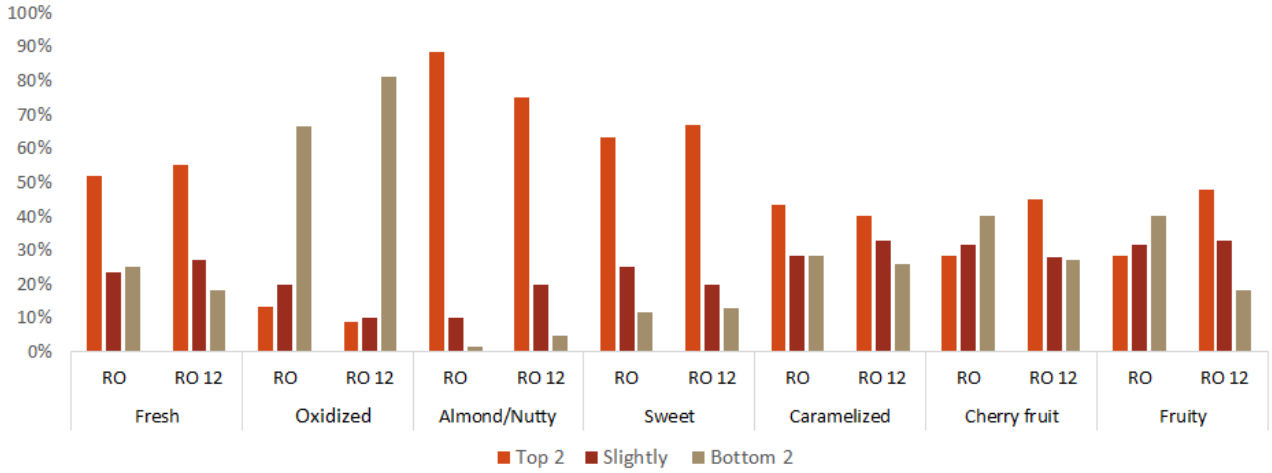

Figure 1: Attributes analyses in roasted pit oil at Time 0 (RO) and after 12 weeks of storage (RO12)
Top 2: Very or Moderately Intense, Slightly: Slightly Intense, Bottom 2: Not Very or Not at All Intense.

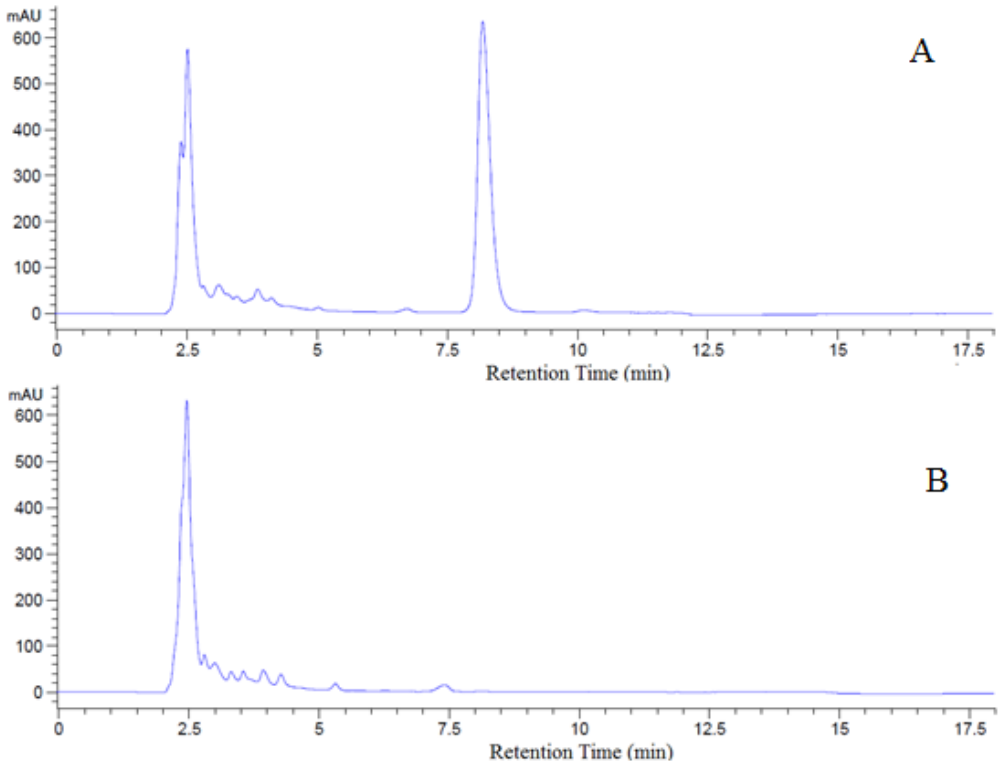

Figure 2: Cherry pit oil chromatograms. (A): Roasted pit oil with spiked amygdalin. (B) Roasted cherry pit oil chromatogram.

APPENDIX 3: MELATONIN RESULTS

Table 1: Recovery rates in melatonin standards.

Standard concentration (µg/g)	Melatonin grams (^{1}H-NMR)	Expected grams	Recovery (Results/ Expected)
100 (1)	0.00012	0.00120	1.17
100(2)	0.00012	0.00120	1.17
100(3)	0.00010	0.00100	1.10
10(1)	0.00001	0.00010	0.93
10(2)	0.00001	0.00012	1.05
10(3)	0.00001	0.00009	0.97
1(1)	0.00000	0.00001	1.01
1(2)	0.00000	0.00001	0.84
1(3)	0.00000	0.00001	1.13
0.1(1)	0.00000	0.00000	NQ
0.1(2)	0.00000	0.00000	NQ
0.1(3)	0.00000	0.00000	NQ
0.01(1)	Not run since 0.1 concentration was not quantifiable.		
0.01(2)			
0.01(3)			
0.001(1)			
0.001(2)			
0.001(3)			

Table 2: Recovery rates in melatonin supplements.

Matrix	mg of melatonin declared in label	Volume/ mass sampled	grams of melatonin (^{1}H-NMR)	Expected grams	Recovery (Result/ Expected)
Liquid 5 mg (1)	5 mg in 15 ml	3 ml	0.0013	0.001	1.30
Liquid 5 mg (2)		3 ml	0.0014	0.001	1.40
Liquid 5 mg (3)		3 ml	0.0014	0.001	1.40
Tablets 3 mg (1)	3 mg in 1 tablet (diluted in 30 g of water)	10 ml	0.0011	0.001	1.10
Tablets 3 mg (2)		10 ml	0.0012	0.001	1.20
Tablets 3 mg (3)		10 ml	0.0010	0.001	1.00
Liquid 1 mg (1)	1 mg in 4 ml	4ml	0.0014	0.001	1.36
Liquid 1 mg (2)		4ml	0.0014	0.001	1.37
Liquid 1mg (3)		4ml	0.0014	0.001	1.38
Montmorency Juice (1)	NA	10 g	ND	ND	ND
Montmorency Juice (2)		10 g	ND	ND	ND
Montmorency Juice (3)		10 g	ND	ND	ND
Balaton Juice (1)	NA	10 g	ND	ND	ND
Balaton Juice (2)		10 g	ND	ND	ND
Balaton Juice (3)		10 g	ND	ND	ND
Processed Juice (1)	NA	10 g	ND	ND	ND
Processed Juice (2)		10 g	ND	ND	ND
Processed Juice (3)		10 g	ND	ND	ND

APPENDIX 4: CHERRY PIT OIL – BUSINESS CASE

Goal

Determine the feasibility of production of cherry pit oil in New York State (NYS).

Participants

- Cherry Juice Producers
- Distributors
- Retailers

Roasted cherry pit oil profile

- **Quality:** Similar color to sesame seed oil. Lower peroxide values (0.99-1.36 meq O_2/ kg), than results previously reported (1.6 and 2.8 meq O_2/ kg) by Popa et al. (2011) and Özcan et al. (2015)
- **Safety:** No amygdalin detected
- **Nutritional:** Oleic and Linoleic are the predominant Fatty Acids. Erucic Acid is also present
- **Sensory:** 53% of the participants liked the aroma of the oil. After 12 weeks of storage people liked the oil in the same way (56% liked it). More than 60% of the participants also liked the color of the oil. The predominant Aroma attributes identified were Almondy and Sweet. The Caramelized and Roasted Aromas were also important attributes in it.

SWOT Analysis

Strengths

- Significant amount of pits disposed yearly due to the cherry juicing processing industry
- No Cherry Pit Oil available in the market
- Specialty Oils market is increasing
- Good Sensory, Quality and Nutritional Properties

Weaknesses

- Variability on quality of the Cherry Pits
- Supply Uncertainty: Availability of cherry pits depend on juice demand and factors such as weather conditions
- Oil Yield is low (4-5% Dry Basis)

Opportunities

- Expand to other cherry producing states such as Michigan, Utah, Washington and Wisconsin
- Different applications of the oil depending on the pits conditioning treatment: Flavoring Agent, Cosmetic Industry, others

Threats

- Similar products are being launched: almond oil (however, this one is deodorized so it has no distinctive flavor) and apricot kernel oil (used for cosmetic industry)
- Skepticism to accept a product made of by-products

Cherry pits supply

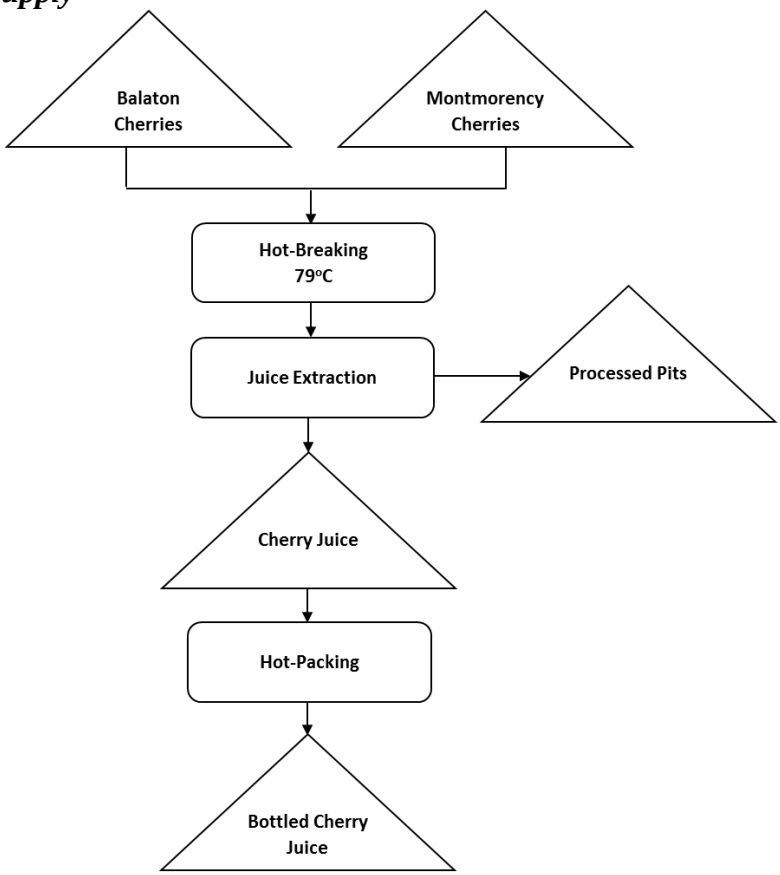

Figure 1: Cherry juicing process. Pits for cherry pit oil production are obtained from this process.

Roasted cherry pit oil operation

Roasting time may increase by 1 or 2 minutes in the real operation in order to improve moisture content and obtain a better yield

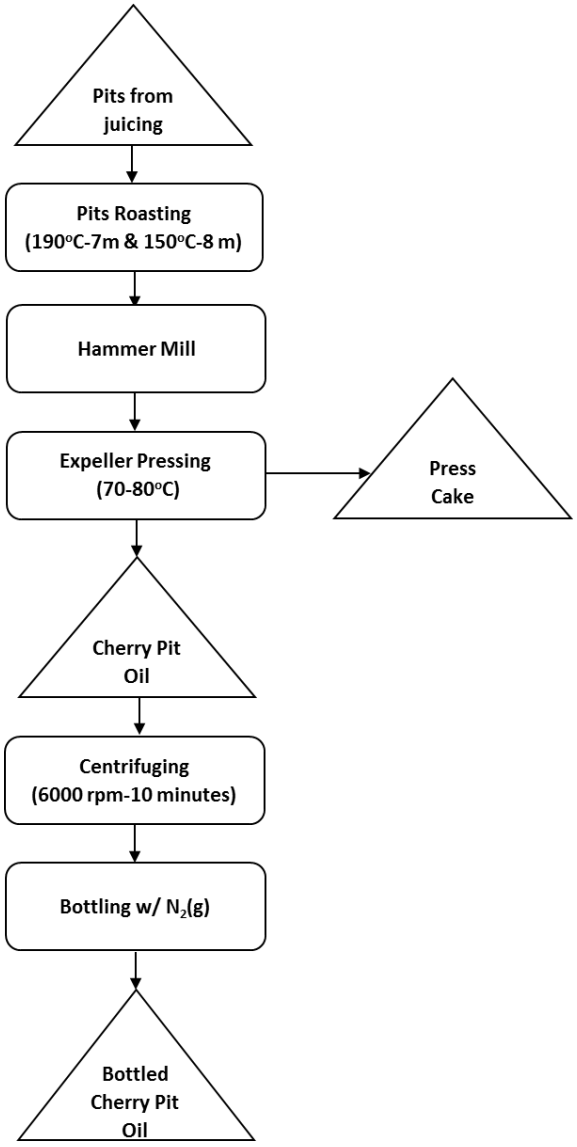

Figure 2: Cherry pit oil production.

Pits availability in NYS

Table 1: Production of Tart Cherries in 2015 and 2016 and Forecast 2017[(1)].

Year	Tart Cherries Produced in United States in 2016 (Million Pounds)	Tart Cherries Produced in NYS in 2016 (Million Pounds)	% Tart Cherry Production in NYS
2015	252.5	10.5	4.16
2016	309.1	8	2.59
Forecast 2017	238.2	9	3.78
Average	**266.6**	**9.2**	**3.51**

Table 2: Distribution of Tart Cherries- Industry Uses[(2)].

Distribution of Tart Cherries	
99% processed	
Distribution within Processing	
Frozen	71%
Canned	22%
Juice/ Wine/ Brine	7%
Assumed that 5% goes only for Juicing	

Table 3: Cherries and Pits available annually in NYS**.**

Average Cherry Production in NYS (Million Pounds)[(1)]	Million Pounds of cherries processed (99% of total)[(2)]	Million Pounds of cherries used for Juicing (5% of total processed)[(2)]	Ratio pit/ cherry (90 samples of each cultivar)[(3)]	Pits available yearly from juicing process (Million Pounds)	Pits available yearly from juicing process (Pounds)	Pits available yearly from juicing process (kg)
9.2	9.108	0.455	0.1506	**0.0686**	**68583**	**31109**

Table 4: Cherries and Pits available annually in NYS.

Pits available yearly from juicing process (Kg)	Liters of oil that can be produced annually (Yield=2.3-3.0 % v/m, in fresh basis)[(4)]	Bottles of oil that can be produced annually (250 ml/ bottle)
31109	716-934	2862-3733

Conclusions

- From the 9.2 Million Pounds Tart Cherries produced in New York State, 99% are processed. Specifically, within the processing category 5% are utilized for the juicing industry.
- An average of 0.455 Million Pounds (or 228 US Tons) of Cherries used for juicing give a total of 68583 pounds (or 31109 kg) of pits that can be used for the cherry pit oil industry.
- The Yield expressed as grams of oil obtained for every 100 grams of fresh pits is between 2.3 and 3%.
- Considering the yield and the amount of pits available in NYS, on average 825 Bottles can be produced annually

Profitability

Assumptions

- Data from the previous tables are used.
- Tart cherries used for juicing: 0.445 Million Pounds (228 US ton).
- Average number of bottles produced annually in NYS: 3300 bottles of 250 ml.
- Average Costs and Investments were taken from the Grape Seed Oil business case[(5)]. However, modifications in the final retail price, Company selling price and certain costs and investment were adjusted.
- Final retail selling price is calculated on the price of specialty oils.

Case 1: Three different cherry juice producers will use all their cherry pits to produce cherry pit oil.

Table 5: Pricing and Breakeven Point Analysis for Case 1.

Data Available from Previous Tables and Price Estimate		**Estimated Costs**		**Estimated Profit**	
Ton of whole cherries for juicing/ Year	228	**Man hours cost (Cost 1)**		Company Income ($)/ ton cherries	130
Liters/ ton of cherries	4	Man hours/ton	1	Total Cost ($)/ ton cherries	62
Total Liters of Oil/ Year	825	$/man hour	12	Profit ($)/ ton cherries	68
Total Bottles (250 ml)/ Year	3300	$/ton	12	**Total Profit (228 tons of cherries/ year)**	**15473**
Final Retailer selling price (Retailer adds 40% Margin to our selling price)	15	$ for 228 tons	2736		
Company Selling Price (60% of Retail Price)	**9**	**Bottles/ Labels/ Utilities (Cost 2)**		**Estimated Investment**	
Company Income per 3300 bottles (1 Year)	**29700**	$/ton	20	Press/ Roaster/ hammer mill/ etc ($)	25000
		$ for 228 tons	4560	Profit per year approximately	15400
		Pick-up/ delivery Pits (Cost 3)		**Breakeven Point (years)**	**1.6**
		$/ton	20		
		$ for 228 tons	4560		
		Production Cost (Cost 1+ Cost 2+ Cost 3)			
		$/ton	52		
		$ for 228 tons	11856		
		Distribution Cost (20% Prod Cost)			
		$/ton	10		
		$ for 228 tons	2371		
		Total Cost (Cost 1 + Cost 2 + Cost 3 + Distribution)			
		$/ton	62		
		$ for 228 tons	**14227**		

Case 2: One of the three cherry juice producers decides to produce its own cherry pit oil with the pits from its own facility - which is 1/3 of the total volume produced in NYS.

Table 6: Pricing and Breakeven Point Analysis for Case 2.

Data Available from Previous Tables and Price	
Tons of whole cherries for juicing/ Year	76
Liters/ ton of cherries	4
Total Liters of Oil/ Year	275
Total Bottles (250 ml)/ Year	1100
Final Retailer selling price (Retailer adds 40% Margin to our selling price)	15
Company Selling Price (60% of Retail Price)	**9**
Company Income per 1100 bottles (1 Year)	**9900**

Estimated Costs	
Man hours cost (Cost 1)	
Man hours/ton	2
$/man hour	12
$/ton	24
$ for 76 tons	1824
Bottles/ Labels/ Utilities (Cost 2)	
$/ton	20
$ for 76 tons	1520
Pick-up/ delivery Pits (Cost 3)	
N/A (Uses its own pits)	
Production Cost (Cost 1+ Cost 2+ Cost 3)	
$/ton	44
$ for 76 tons	3344
Distribution Cost (20% Prod Cost)	
$/ton	9
$ for 76 tons	669
Total Cost (Cost 1 + Cost 2 + Cost 3 + Distribution)	
$/ton	53
$ for 76 tons	**4013**

Estimated Profit	
Company Income ($)/ ton cherries	130
Total Cost ($)/ ton cherries	53
Profit ($)/ ton cherries	77
Total Profit (76 tons of cherries/ year)	**5887**
Estimated Investment	
Press/ Roaster/ hammer mill/ etc ($)	15000
Profit per year approximately	5800
Breakeven Point (years)	**2.6**

Conclusions

- In both scenarios the operation is profitable
- In the first case, the breakeven point is achieved 1 year faster. However in both cases the breakeven point is achieved in less than 3 years
- In Case 1 the man hour is cheaper (economy of scale), but in the second case there are no costs of transportation associated with getting the pits from all factories to the cherry pit oil producing facility
- If the demand of tart cherry juice continues to be stable, the supply of cherry pits will also be stable. If this is the case, investing in this process will be promising

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(1) USDA (2017). Cherry Production. Retrieved From: http://usda.mannlib.cornell.edu/usda/current/CherProd/CherProd-06-09-2017.pdf

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(3) Obtained from own research measurements of 90 Balaton Samples and 90 Montmorency Samples.

(4) Obtained from own research measurements of Yield in the Pressing Process

(5) Sustainable Agriculture Research Association. Grapeseed Oil Research Project for Sustainable Viticulture. Retrieved from: http://www.weigga.org/GSO_Power_Point_IWGA.pdf